

THE ROLE OF THE INOSITOL PHOSPHATASE, SHIP,
IN THE INNATE IMMUNE RESPONSE TO
SALMONELLA TYPHIMURIUM

by

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ABSTRACT

The SH2 domain-containing inositol 5'-phosphatase, SHIP, negatively regulates hematopoietic cell functions and is critical for maintaining immune homeostasis. However, whether SHIP plays a role in controlling bacterial infections *in vivo* remained unknown. *Salmonella enterica* causes human salmonellosis, a disease that ranges in severity from mild gastroenteritis to severe systemic illness, resulting in significant morbidity and mortality worldwide. The focus of this work was to determine the role of SHIP in a murine model of systemic Salmonellosis. Susceptibility of *ship*^{+/+} and *ship*^{-/-} mice to *S. enterica* serovar Typhimurium infection was compared. *ship*^{-/-} mice displayed an increased susceptibility to both oral and intraperitoneal *S. Typhimurium* infection and had significantly higher bacterial loads in intestinal and systemic sites than *ship*^{+/+} mice, indicating a role for SHIP in the gut and systemic pathogenesis of *S. Typhimurium in vivo*. Blood cytokine levels showed that infected *ship*^{-/-} mice produce lower levels of Th1 polarizing cytokines compared to *ship*^{+/+} animals, and analysis of supernatants taken from M2 bone marrow derived macrophages correlated with this data. M2 macrophages were the predominant population *in vivo* during both oral and intraperitoneal infections. Because M2 macrophages are poor defenders against bacterial infection, these data suggest that M2 macrophage skewing in *ship*^{-/-} mice contributes to ineffective clearance of *Salmonella*.

The role of SHIP in the gut during enteric infections was also explored. *ship*^{-/-} mice were not susceptible to *Citrobacter rodentium* infection, yet

developed severe inflammation of the ileum upon infection with this bacterium, with *Salmonella*, or when challenged orally with LPS. Increased collagen deposition was also observed at early time points post-infection, suggesting that *ship*^{-/-} mice may be used to study the development of inflammatory bowel diseases characterized by fibrosis, such as Crohn's.

Because SHIP is such a critical negative regulator in both innate and adaptive immune cells, it has the potential to significantly alter the outcome of infections. This work highlights the fact that SHIP is important *in vivo* during Salmonellosis and opens new avenues to explore targeting SHIP in therapies for both systemic infections as well as inflammatory bowel diseases.

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LIST OF ABBREVIATIONS

7AAD	7-amino-actinomycin D
apc	allophycocyanin (flow cytometry)
APC	antigen presenting cell
BPI	bacterial permeability inducing protein
BMDM	bone marrow-derived macrophage
CAMP	cationic antimicrobial peptide
CBA	cytometric bead array
CD	Crohn's disease
CTGF	collagen transforming growth factor
DAPI	4', 6-diamidino-2-phenylindole
DC	dendritic cell
DMEM	Dulbecco's Modified Eagle Medium
ECL	enhanced chemiluminescence
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme-linked immunoabsorbant assay
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
GM-CSF	granulocyte colony stimulating factor
HBSS	Hank's balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	horseradish peroxidase
IBD	inflammatory bowel disease
IF	immunofluorescence
IFN	interferon
IHC	immunohistochemistry
IL	interleukin
IP	intraperitoneal
LB	Luria-Bertani
LPS	lipopolysaccharide
M ϕ	macrophage
MCP	monocyte chemotactic protein
M-CSF	monocyte colony stimulating factor
MLN	mesenteric lymph nodes
MOSF	multiple system organ failure
NGS	normal goat serum
NK	natural killer
NO	nitric oxide
NTS	non-typhoidal <i>Salmonella</i>
OMP	outer membrane protein
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
PE	phycoerythrin
PFA	paraformaldehyde

PI3K	phosphatidylinositol 3' kinase
PIP	phosphatidylinositol
PMN	polymorphonuclear cell
PRR	pattern recognition receptor
PTEN	phosphatase and tensin homologue deleted chromosome 10
RADIL	Research Animal Diagnostic Laboratory
ROS	reactive oxygen species
RNS	reactive nitrogen species
SA	streptavidin
SCV	<i>Salmonella</i> -containing vacuole
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH2	src homology 2
SHIP	src homology 2 domain-containing 5'inositol phosphatase
SI	small intestine
SPI	<i>Salmonella</i> pathogenicity island
TLR	toll-like receptor
TGF	transforming growth factor
TNF	tumor necrosis factor
TTSS	type three secretion system
UC	ulcerative colitis
WB	Western blot

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DEDICATION

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EY,

Jenna

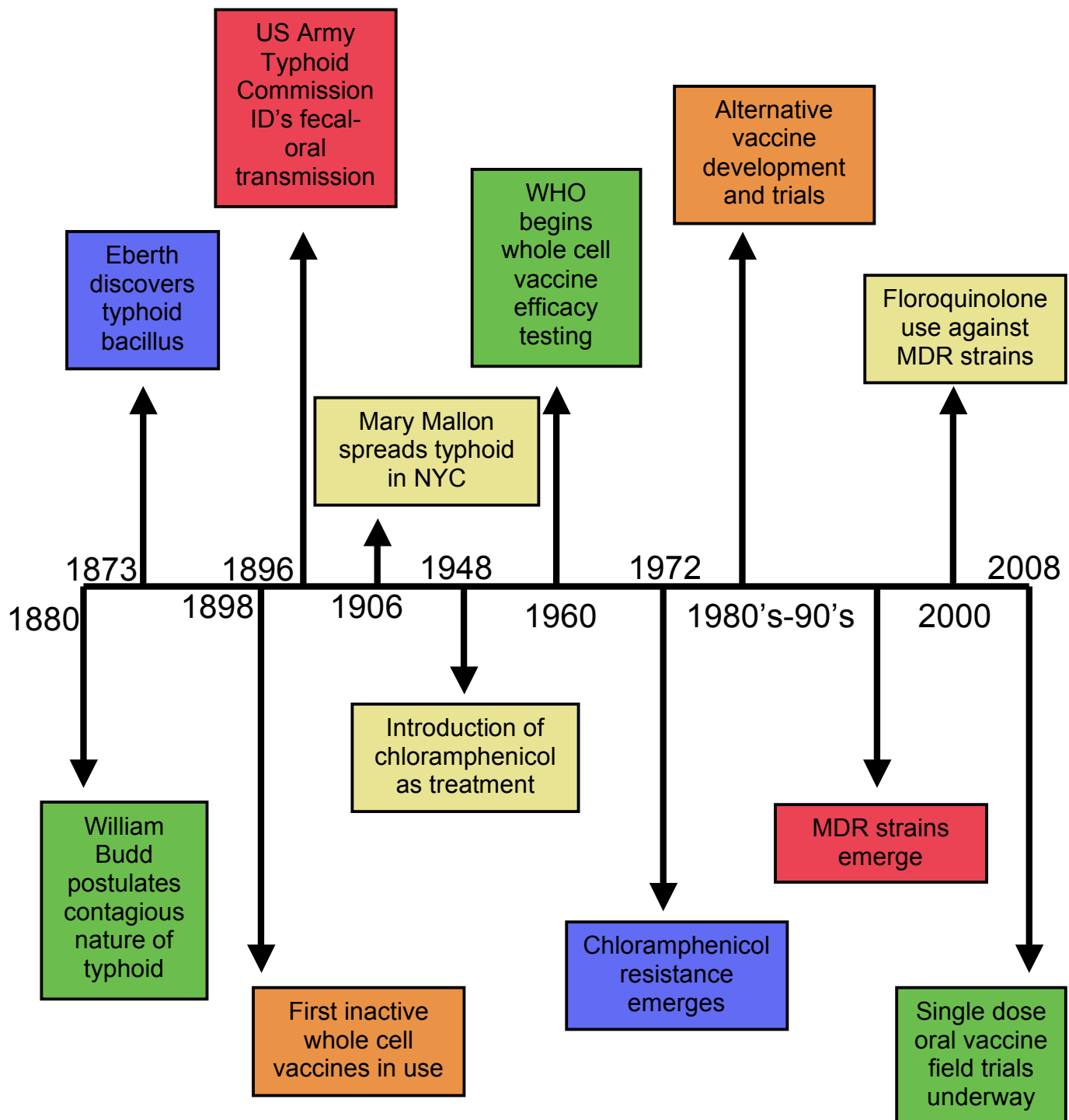
CHAPTER 1: INTRODUCTION

1.1 History and epidemiology of salmonellosis

Infectious diseases have played a central role in the development of human civilizations since ancient times. Infamous epidemics of plague, smallpox, cholera and influenza have tipped the balance of economies, militaries, politics and populations at the most critical points in world history. In an era of medical miracles and scientific innovation, it is easily forgotten what a profound effect infectious diseases have in the global community. While AIDS often takes center stage as the world's most serious health crisis, other communicable diseases, like enteric fevers and food-borne illness, cause severe morbidity and mortality in millions of people on a daily basis.

Enteric fevers are caused by the human-specific pathogen *Salmonella enterica* serovars Typhi and Paratyphi. Exposure via contact with contaminated water and food leads to systemic dissemination of bacteria and resulting disease characterized by high fever, general malaise and flu-like symptoms. During the 19 and 20th centuries, typhoid fever was a common household illness found in every corner of the globe and was often indistinguishable from other sicknesses with similar symptoms, such as malaria, dengue fever and typhus (Mastroeni and Maskell, 2006). However, with the advancement of microbiological techniques in the late 1800's, contagions and their sources were identified and detailed studies of *Salmonella* bacteriology and treatment options were explored (Fig. 1).

Figure 1. History and treatment of salmonellosis



Today, enteric fevers are most common in the developing world; typhoid is endemic in Asia and Africa, and is a frequent cause of disease in the Middle East, South America and even parts of Southern and Eastern Europe (Mastroeni and Maskell, 2006). Globally, there are an estimated 22 million cases of typhoid fever each year and roughly 10% of these cases are fatal (Crump *et al.*, 2004). High as these numbers are, they do not include the billions of individuals affected by non-typhoidal salmonellosis (NTS), a self-limiting gastroenteritis typically associated with food poisoning. Like typhoid fever, NTS is spread via the fecal-oral route but is caused by infection with various *S. enterica* serovars. These include Typhimurium, Enteritidis, Newport and Heidelberg, all of which have reservoirs in animal populations used for agriculture (Hohmann, 2001; Rabsch *et al.*, 2001). As such, these pathogens are among the most important linked to food-related deaths (Kennedy *et al.*, 2004). Estimates for numbers of NTS cases worldwide vastly exceed those for enteric fevers; there are 1.3 billion cases annually, 300,000 of which are fatal (Pang *et al.*, 1995). While a significant amount of cases are still seen in developed countries like the US, Africa bears the brunt of this burden. For example, NTS is the most common cause of bacteremia in children in Africa, and NTS infection rates can reach up to 60% of HIV/AIDS patients there (Gordon MA, 2004; Graham, 2002).

Both typhoid fever and NTS are contracted by ingestion of *S. enterica* serovars present in contaminated food or water sources. While NTS associated serovars are typically introduced to meat and dairy products from their hosts during processing, *S. Typhi* is shed in feces or urine from people suffering from typhoid fever, or chronic carriers that show no symptoms of disease. Most *S. enterica* are quite robust in the environment, being able to survive even in ice, dust or raw sewage for prolonged periods of time (Mastroeni and Maskell, 2006). As such, risk factors for contracting both enteric fevers and NTS are much the same as those identified in the late 20th century-poor hygiene and limited access to sanitized food and water are the main prerequisites for infection. More recent studies have shown that other socio-economic and environmental factors, such

as poor housing, inadequate food, and rainfall amounts correlate with disease (Gasem *et al.*, 2001; Luby *et al.*, 1998).

1.2 Typhoid infections and treatment options

Infectious doses of *S. enterica* leading to NTS or typhoid fever have a wide range; ingestion of anywhere from 10-100 bacteria has been associated with food poisoning outbreaks and typically 10^3 - 10^9 *S. Typhi* are sufficient to cause fever (Mastroeni and Maskell, 2006). Bacteria are swallowed and many resist the harsh environment of the stomach to adhere to, and invade, the epithelium of the small intestine. At this point, the systemic nature of typhoid becomes apparent and distinguished from most NTS infections, as bacteria disseminate to lymphoid follicles, mesenteric lymph nodes (MLN) and liver within phagocytes of the immune system such as macrophages and dendritic cells (DC). After an incubation period of 7-14 days, secondary bacteremia develops and infection spreads to other organs such as the bone marrow and gall bladder (House *et al.*, 2001). During this time, infected patients will develop the common symptoms associated with febrile diseases, such as high fever, chills, malaise and cough. However, if left undiagnosed or untreated, typhoid can progress and serious complications can result, such as gastrointestinal bleeding or perforation, shock, coma, meningitis and encephalitis (Mastroeni and Maskell, 2006). Even without complications, recovery from untreated typhoid can take up to four months and relapses are not uncommon. It has been estimated that up to 10% of patients suffer relapsed infections and between 1-4% of typhoid sufferers will become chronic carriers of the bacteria for up to one year (Caygill *et al.*, 1994; Marmion *et al.*, 1953). Carriage is a serious complication of typhoid infection not only because of the increased risk for transmission, but also because it predisposes patients to bowel, gall bladder and pancreatic cancers (Caygill *et al.*, 1994).

Diagnosis and treatment of typhoid, especially in endemic areas, are difficult for a number of reasons. Firstly, typhoid is not easily distinguished from other diseases common in endemic areas, such as dengue, malaria or

leishmania, except via blood culture of *S. Typhi*. This requires that patients have access to timely diagnostic laboratory tests, which may not be readily available (Butler *et al.*, 1978). In addition, serological tests may not prove that a patient is currently infected with *S. Typhi*; the presence of anti-*S. Typhi* antibodies from prior infections, vaccinations or cross-reactive epitopes can easily skew the tests towards a false-positive result (Parry *et al.*, 1999). If properly diagnosed, treatment with classic antibiotic regimens is becoming increasingly difficult since resistance to many of the drugs once used to combat typhoid, such as chloramphenicol, amoxicillin and ampicillin, is widespread (Fig. 1). However, with careful assessment of resistance on a regional level, classic antibiotics and fluoroquinolones can still be effective against susceptible bacteria. In fact, fluoroquinolone treatment can cure up to 96% of typhoid cases and reduce carriage to lower than 2% (Gulig *et al.*, 1993; Parry, 2004).

With increasing resistance to fluoroquinolones and limited access to clean water, as well as sanitary agriculture and animal husbandry practices in developing nations, it is essential that more efforts go towards prevention rather than treatment of Salmonellosis. Vaccinology therefore, has been a prime target for controlling the spread of *Salmonella* in both humans and livestock. Whole cell inactivated vaccines against typhoid have been used in humans since the late 1800's (Fig. 1) but their deleterious side-effects prevented widespread use (Engels *et al.*, 1998). Since then, development of more efficacious methods has been an ongoing struggle because i) we have only limited knowledge of immunity and memory to systemic typhoid, ii) many vaccine candidate mutants are either under or over-attenuated, iii) there is little cross-protection for one vaccine against other serovars and strains causing enteric fevers and NTS, and iv) economic pressures often prevent the implementation of vaccine programs (Boyle *et al.*, 2007). Despite these obstacles, two typhoid vaccines have been licensed, the Ty21A live-attenuated vaccine and the Vi antigen subunit vaccine. Because efficacy of these vaccines ranges from 42-92% and they work on a multiple dosing regimen, projects to develop better vaccines are ongoing (Mastroeni and Maskell, 2006). Most promising is a live attenuated $\Delta aroC/ssaV$

oral vaccine that can be administered in a single dose. This vaccine has been trialed successfully in the west and the positive results are being verified in both adults and children living in typhoid endemic areas (Boyle *et al.*, 2007; Stratford, 2005).

1.3 Animal models of salmonellosis

Because *S. Typhi* is host-restricted to humans, an animal model system to study typhoid fever must utilize other *S. enterica* species that have a broader host range yet cause similar symptoms of disease. Why host restriction occurs is a matter of debate, but factors such as host environment as well as genetics of the host and infecting strain most likely play key roles (Mastroeni and Maskell, 2006). Conveniently, *S. Typhimurium*, one of the major strains associated with NTS, can cause systemic disease in mice that mimics human typhoid. Depending on the expression of the *Slc11a1* (*Nramp1*) gene, mice are either genetically resistant or susceptible to systemic *S. Typhimurium* infection, allowing researchers to study both acute and persistent forms of the disease (Blackwell *et al.*, 2001). This animal model has been an invaluable tool for the study of *Salmonella* pathogenesis and the immune responses required to clear infection.

The classic murine model of *S. Typhimurium* infection mimics the systemic disease seen in human typhoid fever and is not a model for the gastroenteritis associated with food poisoning or NTS infections. Bacterial dissemination does not induce enterocolitis or diarrhea in mice, but the animals exhibit classic inflammation at infection foci throughout the body (Santos *et al.*, 2001). To study intestinal disease in relation to *S. Typhimurium*, two models are commonly used. The first is the more classic model where calves infected with *S. Typhimurium* develop enterocolitis that closely resembles human disease (Santos *et al.*, 2001). More recently however, a new *S. Typhimurium* murine model has been developed that also mimics *Salmonella* induced human enterocolitis. It has been shown that mice treated with the antibiotic streptomycin prior to infection with *S. Typhimurium* develop severe intestinal pathology, characterized by edema and infiltration of inflammatory cells. While classical diarrhea is still not induced in

streptomycin-treated mice, and inflammation is limited to the caecum and colon compared to the small intestinal inflammation seen in humans, it is a large step forward in the search for more relevant animal models used to study *Salmonella* pathogenesis (Barthel *et al.*, 2003).

1.4 *Salmonella* pathogenesis in murine typhoid

Using the mouse model of systemic typhoid, we have been able to discern specific events key to the pathogenesis of *Salmonella* and the immune response to infection. As in humans, ingestion of bacteria allows for interaction with intestinal epithelium. Phagocytic M cells located in the Peyer's patches of the small intestine are seen as the canonical method of entry by which *Salmonella* penetrates the gut to gain entry to systemic sites (Jepson and Clark, 2001). As well, *Salmonella* can invade enterocytes and be sampled from the lumen by DCs (Rescigno *et al.*, 2001). Systemic spread occurs as *S. Typhimurium* survives within macrophages (Salcedo, 2001), DCs (Richter-Dahlfors *et al.*, 1997), polymorphonuclear cells (PMNs) (Sheppard *et al.*, 2003), and B Cells (Yrlid *et al.*, 2001) in various organs such as the MLN, spleen and liver. Contradictory to many observations of *S. Typhimurium* replication in cell culture systems, *Salmonella* does not replicate to high numbers per cell *in vivo* and infection foci grow in number rather than in sheer size (Vazquez-Torres *et al.*, 1999). Furthermore it is clear that at late time points in infection *Salmonella* can exist in the blood in CD18+ cells (Richter-Dahlfors *et al.*, 1997; Sheppard *et al.*, 2003) or extracellularly. Depending on the dose used in the infection and the susceptibility of the mouse strain, immune responses will dictate whether systemic bacteria are cleared or replicate to high enough numbers to cause bacteremia and death (Fig. 2).

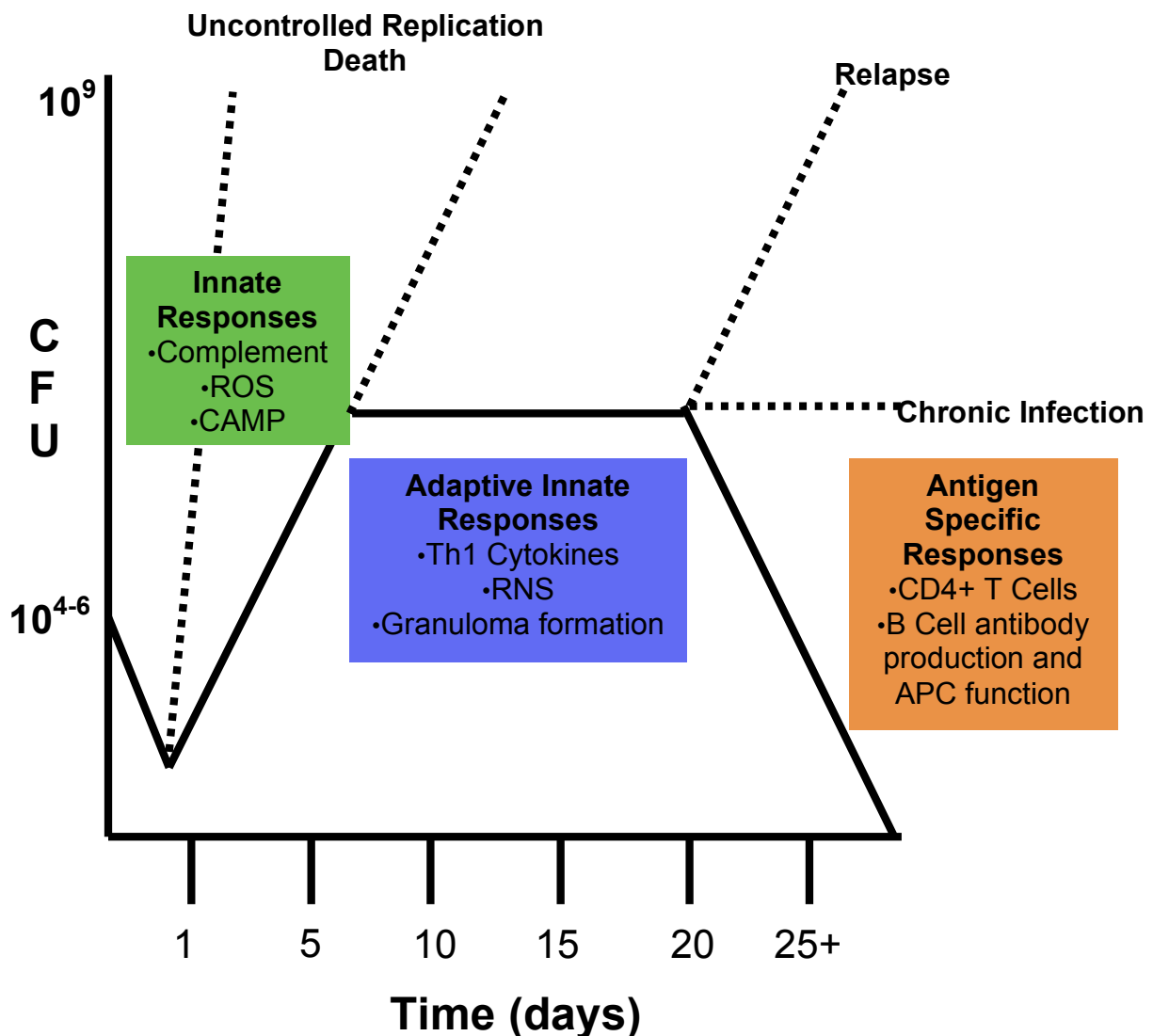
During the invasion of the gut and spread to systemic sites, *Salmonella* uses a variety of virulence factors for pathogenesis. While virulence genes can be found anywhere in the genome, in *Salmonella* they are mostly clustered on Pathogenicity Islands (SPI). *Salmonella* has 10 SPIs, the first two of which are most fully characterized in the typhoid infection model. Encoded on SPI-1 and 2

are type-three secretion systems (TTSS) that are essential for *Salmonella* pathogenesis. These protein complexes act as "molecular syringes" that inject bacterial encoded proteins, termed effectors, directly into host cells. These effectors modulate cell signaling that promotes either bacterial invasion of host cells (SPI-1 effectors) or intracellular survival and systemic spread of bacteria (SPI-2 effectors) (Gal-Mor and Finlay, 2006; Hensel, 2004). For example, SPI-1 encoded effectors are necessary to induce cytoskeletal rearrangements required for entry into non-phagocytic cells (Schlumberger and Hardt, 2006) and are only required for colonization of the intestinal tract, but not systemic spread, in the mouse typhoid model (Galan and Curtiss, 1989). In contrast, SPI-2 effectors are required for the maintenance of, and replication within, the *Salmonella* Containing Vacuole (SCV), the specialized compartment *Salmonella* form when phagocytosed that prevents degradation by the normal endocytic pathway (Galan, 2001) . In addition, effectors of both SPI-1 and SPI-2 encoded TTSS are essential for modulating host immune responses against *Salmonella* (Table 1).

Table 1. *Salmonella* secreted effectors and their function in host cells

Location	Effector	Target Cell	Function	Reference
SPI-1	SipA	Epithelial	Invasion Inflammation	(Zhang <i>et al.</i> , 2003)
SPI-1	SipB	Epithelial PMN MΦ	Invasion Recruitment Apoptosis, increased IL-1β	(Monack <i>et al.</i> , 2000)
Chrom.	SopA	Epithelial PMN	Invasion Recruitment	(Raffatellu <i>et al.</i> , 2005; Wood <i>et al.</i> , 2000; Zhang <i>et al.</i> , 2006)
SPI-5	SopB (SigD)	Epithelial PMN MΦ	Invasion Recruitment Prevents lysosomal fusion	(Dukes <i>et al.</i> , 2006; Raffatellu <i>et al.</i> , 2005; Zhang <i>et al.</i> , 2002; Zhou, 2001)
Chrom. Phage	SopD/E/ E2	Epithelial PMN	Invasion Recruitment	(Bakshi <i>et al.</i> , 2000; Raffatellu <i>et al.</i> , 2005; Zhang <i>et al.</i> , 2002)
SPI-1	SptP	Epithelial	Decreased IL-8	(Haraga and Miller, 2003)
Gifsy-3 Prophage	Ssph1	Epithelial	Decreased NF-κβ signaling	(Haraga and Miller, 2003)
Chrom. SPI-2 SPI-2 Chrom.	SifA SseF SseG PipB2	MΦ Epithelial	Sif formation SCV integrity Intracellular survival	(Beuzon <i>et al.</i> , 2000; Knodler and Steele-Mortimer, 2005; Kuhle and Hensel, 2002; Stein <i>et al.</i> , 1996)
SPI-2	SpIC	Phagocytes	Vesicular trafficking Intracellular survival	(Uchiya <i>et al.</i> , 1999)

Figure 2. Immune responses to *S. Typhimurium* *in vivo*. Modified from *Salmonella* infections : clinical, immunological and molecular aspects. Mastroeni and Maskell, 2006. Solid lines represent systemic CFU trends in *Salmonella* resistant mouse strains. From 0-5 days innate responses are responsible for initial decreases in bacterial load and controlling bacterial replication. Adaptive responses have a bacteriostatic effect between days 5-20. During relapses or chronic infections (dashed lines), antigen specific responses are required to reduce bacterial load. If any immune response is impaired, bacteria replicate to high numbers (dashed lines), leading to death (days 1-10 post-infection) or relapsed and chronic infections.



1.5 Immunity against *Salmonella* in murine typhoid

Immunity against *S. Typhimurium* during murine typhoid infection can be separated into three distinct types of immune responses, innate, adaptive/innate and antigen-specific (Fig. 2). Innate immunity to *Salmonella* is required for initial bactericidal actions against colonizing and invading bacteria and is most important in the first days of infection. It begins immediately in the digestive tract with the production of non-specific humoral defenses such as cationic antimicrobial peptides (CAMPs) present in the saliva and gut, as well as opsonins of the complement cascade (Warren *et al.*, 2002; Wehkamp *et al.*, 2007). Once bacteria invade past the epithelium, phagocytes, especially macrophages and DCs, recognize *Salmonella* via interactions between their surface pattern recognition receptors (PRRs) and pathogen-associated molecular patterns (PAMPs) on the bacterial surface. Importantly for *Salmonella* recognition is the interaction between CD14/TLR4 complexes on the host cell surface and bacterial lipopolysaccharide (LPS). This induces bacterial phagocytosis into the cell, as well as the production of various microbicides, such as acids, enzymes, reactive oxygen and nitrogen species (ROS/RNS), that degrade bacteria within the endocytic pathway (Rosenberger and Finlay, 2002; Vazquez-Torres *et al.*, 2000a). While *Salmonella* have SPI-2 encoded mechanisms to defend against ROS as well as phago-lysosome fusion (Table 1), these molecules are essential for the rapid killing of bacteria by the cell and protection from infection *in vivo* (Mastroeni, 2000; Vazquez-Torres *et al.*, 2000b).

Central to the ability of phagocytes to initiate these innate responses against *Salmonella* in mice is the expression of the Slc11a1 (previously known as Nramp1) gene that codes for a divalent cation transporter that localizes to the membrane of phagosomes within macrophages, DCs and PMNs during *Salmonella* infections (Bellamy, 1999). While it is known that susceptibility of mice to typhoid infection is dependent upon the expression of the dominant Slc11a1 resistant allele, why this occurs is a matter of debate. This is because it is unknown whether the function of Slc11a1 is to change the availability of nutrients, such as iron and manganese, within the phagosome by moving ions in,

out, or in either direction (Forbes and Gros, 2003; Goswami *et al.*, 2001; Kuhn *et al.*, 1999). The expression of this transporter is essential for preventing the replication of *Salmonella in vitro* as well as in PMNs of the liver and spleen in the mouse model (Forbes and Gros, 2001; Richter-Dahlfors *et al.*, 1997). In humans Slc11a1 expression does not seem to control susceptibility to typhoid infections (Dunstan *et al.*, 2001), even though it is required to combat other intracellular pathogens such as *Mycobacterium tuberculosis* and *Leishmania* (Blackwell *et al.*, 2001).

Recognition of *Salmonella* by macrophages and other professional phagocytes is essential for the induction of the adaptive/innate immune response, which is responsible for bacteriostasis during murine typhoid infections (Fig. 2). The production of several cytokines by macrophages, DCs, PMNs and natural killer (NK) cells is the hallmark of this stage of immunity; these are required for controlling systemic bacterial replication as well as initiating later antigen-specific responses (Table 2). For example, while the classic pro-inflammatory mediator $\text{TNF}\alpha$ is required to restrict bacterial growth to infection foci within organs (Everest, 1998; Mastroeni, 1995) interleukins (IL) 12, 18 and 15 are essential for the production of $\text{IFN}\gamma$ and the subsequent activation of macrophages to produce ROS and RNS (Kagaya *et al.*, 1989). Elimination of any of these cytokines from the mouse during typhoid infection greatly increases susceptibility to *Salmonella* (Stoycheva and Murdjeva, 2004).

Importantly, the cytokine milieu during *Salmonella* infection skews CD4^+ T helper cells to a Th1 response and IL-2 production by these cells is critical to prevent disease (al-Ramadi *et al.*, 2001; Mittrucker *et al.*, 2002; Srinivasan *et al.*, 2004). In addition, Th1 cells, along with CD8^+ cytotoxic lymphocytes, modulate a variety of antigen-specific immune events central to the clearance of *Salmonella* from systemic sites, including B cell antibody production and isotype profile. B cells can make antibodies against various *Salmonella* antigens, including LPS, the capsular Vi polysaccharide, outer membrane proteins (OMPs), flagellin and heat shock proteins (Mastroeni, 2002). While B cells are classically known to play an important role in the memory response to secondary *Salmonella*

infections, they also act during initial infections as antigen presenting cells (APC), which is required for the development of Th1 responses (Mastroeni *et al.*, 2000).

Table 2. Cytokines required for controlling *Salmonella* infection in mice.

Cytokine	Producing Cell	Function	Reference
TNF α	M Φ	Inflammation Lesion formation	(Everest, 1998; Mastroeni, 1995)
IFN γ	M Φ , NK, Neutrophils, T Cells	Activation ROS, RNS production Lysosomal fusion	(Billiau, 1996; Foster <i>et al.</i> , 2003; Jouanguy <i>et al.</i> , 1999; Kagaya <i>et al.</i> , 1989)
IL-12	M Φ , DC	IFN γ production RNS production	(Koch F, 1996; Mastroeni <i>et al.</i> , 1996; Mastroeni <i>et al.</i> , 1998)
IL-18	M Φ	IFN γ production RNS production	(Mastroeni <i>et al.</i> , 1999)
IL-15	DC	IFN γ production	(Eckmann and Kagnoff, 2001; Ferlazzo <i>et al.</i> , 2004; Lucas <i>et al.</i> , 2007)
IL-4	NK1.1 Regulatory T Cell	Decrease M Φ bactericidal and cytokine responses	(Denich <i>et al.</i> , 1993; Enomoto <i>et al.</i> , 1997)
IL-10	M Φ	Decrease M Φ function	(Arai, 1995)
IL-6	M Φ , DC	Enhances PMN killing of bacteria	(Nadeau <i>et al.</i> , 2002)
IL-2	T Cells	Final clearance from organs	(al-Ramadi <i>et al.</i> , 2001)

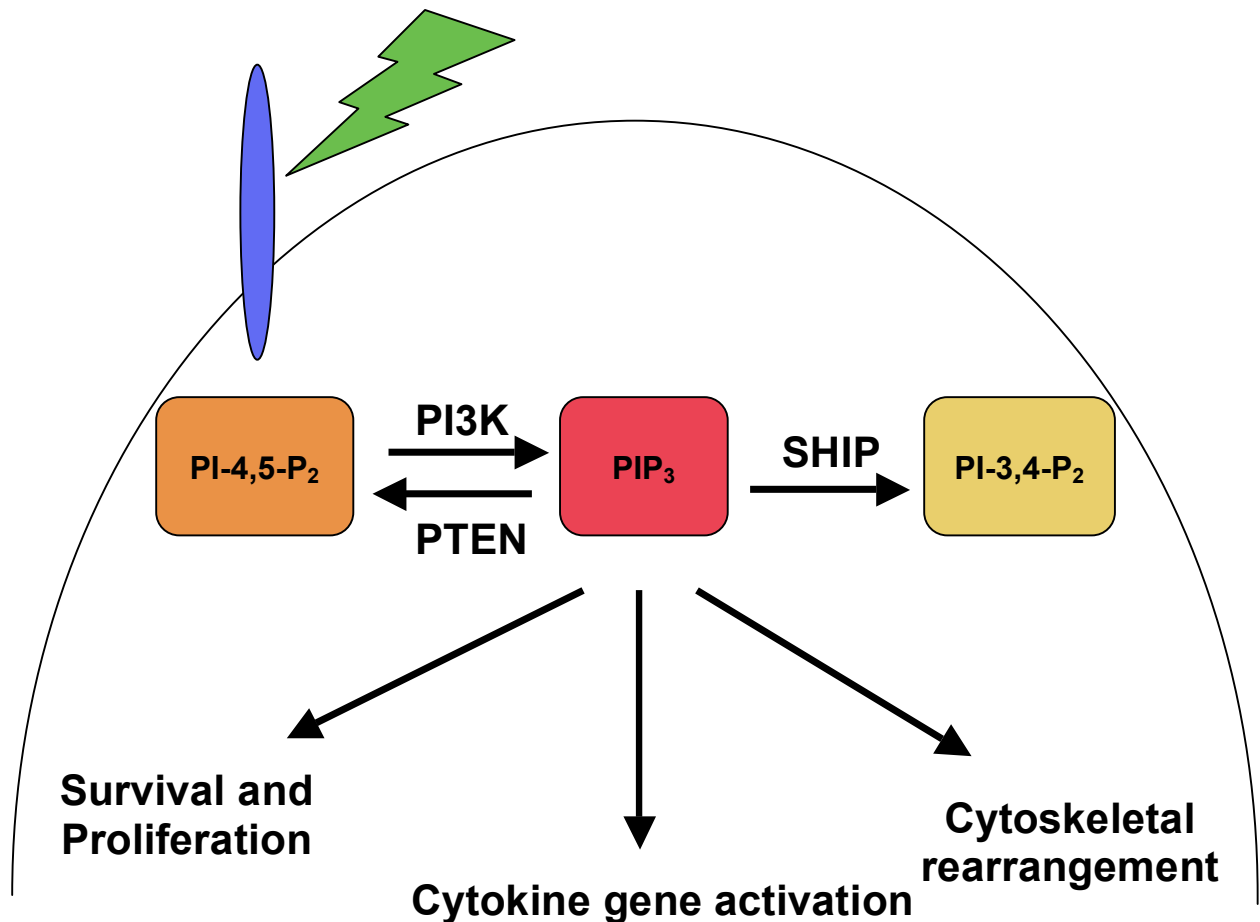
1.6 Negative regulation of immune responses

While the production of potent inflammatory mediators, such as TNF α , IFN γ and ROS, are essential in clearing *Salmonella* infections in humans and mice, severe effects occur if their production is not tightly regulated. Almost 50% of deaths seen in intensive care units in North America are the result of sepsis, which occurs when the intense production of pro-inflammatory products by

macrophages and other cell types in response to bacterial LPS leads to multiple system organ failure (MSOF) and death (West, 2002). Thus, it is clear why all cell types of the immune response have multiple regulatory mechanisms to restrict the duration and intensity of their signals during infections. These mechanisms are complex and involve interactions between immunoreceptors, kinases, phosphatases, ubiquitin ligases, adapter proteins and transcriptional regulators, all of which work together in concert to maintain immune homeostasis (Veillette *et al.*, 2002).

Besides protecting the host from shock, negative regulation of immune responses has also been shown to be pivotal in determining the outcome of pathogenesis for various infectious diseases. For example, the protein tyrosine phosphatase, SHP-1, is a pluripotent regulator of macrophage function that inhibits signaling from cytokine receptors, chemokine receptors, integrins and immunoreceptors, and it is a negative regulator of phagocytosis for both *Neisseria gonorrhoeae* and *Streptococcus suis* (Hauck, 1999; Segura, 2004). In addition, SHP-1 deficiency increases mouse resistance to *Leishmania* infection by increasing macrophage production of NO and inducing macrophage hypersensitivity to IFN- γ (Forget, 2001). In contrast however, it has been found that a lack of SHP-1 is associated with increased susceptibility in mice to viral infections of the central nervous system (Massa, 2002). In addition, loss of TLR regulators, like IRAK-M or A20, led to severe inflammatory disorders (Kobayashi *et al.*, 2002; Lee *et al.*, 2000). As such, it is clear that depending on the pathogen and immune response required to clear infections, negative regulators can have both positive and negative influences on the outcome of pathogenesis.

Figure 3. PI3K signaling and regulation by SHIP



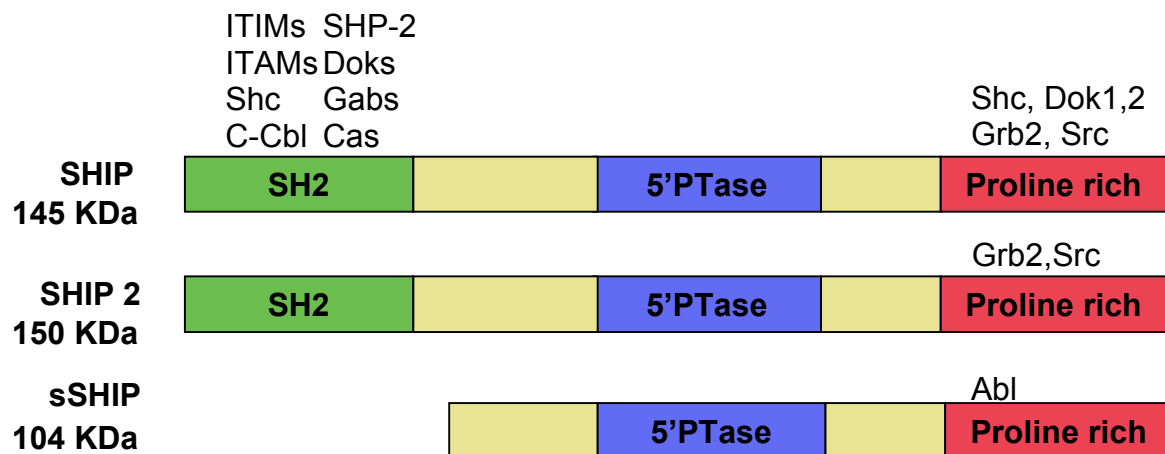
1.7 Regulation of PI3K by SHIP

The phosphoinositide 3-kinase (PI3K) is a key master regulator of signaling in innate immune cells. In response to various stimuli during infections, such as TLR or cytokine receptor ligation, this lipid kinase is immediately targeted to the cell membrane and transfers gamma-phosphate from ATP to phosphoinositides at the 3' position, creating phosphatidylinositol-3,4,5 trisphosphate (PIP₃, Fig. 3). The formation of PIP₃ targets multiple signaling complexes that initiate cell activation. This activated state is characterized by cytoskeletal rearrangements, cytokine production and proliferation (Fig. 3), all of

which are essential in the control of *Salmonella* infection (Hazekei *et al.*, 2007; Krystal, 2000).

Because PIP₃ is such a potent second messenger, there are two distinct mechanisms present in the cell to break the molecule down and thus negatively regulate its signaling capacity. One is the phosphatase and tensin homologue (PTEN), a 53 KDa lipid phosphatase that opposes the function of PI3K and breaks PIP₃ down into PI-4,5-P₂. In contrast, the SHIP family of src-homology 2 domain-containing (SH2) inositol phosphatases hydrolyze PIP₃ into PI-3,4-P₂ (Fig. 3) and also can break down inositol-1,3,4,5 tetrakisphosphate (IP₄) (Krystal, 2000). PTEN is a classic tumor suppressor gene; up to 50% of human cancers, including prostate, breast and lymphomas, are associated with mutations in PTEN, and mice heterozygous for PTEN are prone to tumors. PTEN is thought to prevent malignancies by suppressing the PIP₃-mediated activation of the anti-apoptotic AKT pathway (Cantley and Neel, 1999; Suzuki *et al.*, 1998). The SHIPs however, are less associated with cancers and have been implicated in controlling a wide array of functions in both innate and adaptive immune cells, which will be discussed in further detail below. The redundancy in function between the SHIPs and PTEN underscores the importance of regulating PIP₃ signaling throughout the immune system.

Figure 4. SHIP family proteins. Modified from "The Role of SHIP in macrophages". Sly *et al.*, 2007. A graphic representation of SHIP molecules and domain interactors. Amino terminal src-homology 2 domain (SH2) in SHIP and SHIP2 mediate interactions between these molecules and immuno-tyrosine activating and inhibitory motifs (ITAMs/ITIMs) as well as adapter proteins. A 5' phosphatase (5'Ptase) domain in the center of all SHIPs mediates its inositol phosphatase activity, while proline rich carboxy termini mediate interactions with src-homology 3 (SH3) domain containing adapter proteins.



1.8 SHIP biochemistry and interactors

Three molecules comprise the SHIP family, SHIP, SHIP2 and stem cell, or sSHIP. While SHIP and sSHIP expression are restricted to the hematopoietic and stem cell compartments, respectively, SHIP2 is more ubiquitous and found in most cell types and tissues. SHIPs function by translocating to sites of PIP₃ synthesis in cells stimulated in a variety of ways, such as cytokine or immunoreceptor ligation (March, 2002). The SHIP polypeptide is a 145 KDa molecule, 1190 amino acids long, and is composed of three major domains that characterize its function within immune cells. The amino-terminal SH2 domain of SHIP interacts with phosphorylated immuno-tyrosine activating and inhibitory motifs (ITAMs/ITIMs) present in the cytoplasmic tail of many receptors found on immune cells, such as the Fcε RI IgE antibody receptor as well as the Fcγ RIIβ1 IgG antibody receptor (March, 2002; Ono, 1996; Osborne, 1996). Once

phosphorylated, ITAMs or ITIMs associate with SHIP and consumption of PIP₃ or IP₄ by the central phosphatase domain prevents further downstream cell signaling. In addition, the carboxy-terminus of the molecule mediates multiple interactions between SHIP and other proteins. For example, phosphotyrosines in two NPXY sites are important for SHIP function as they provide binding sites for negative regulator adaptor proteins such as Doks (Lamay, 2000), whereas prolines allow for interactions with SH3 domain containing molecules like Grb (Fig. 4) (Jefferson *et al.*, 1997; Tridandapani *et al.*, 1997). C terminal truncations however, are commonly generated *in vivo* and these isoforms may perform distinct functions (Rohrschneider LR, 2003). sSHIP and SHIP2 share high homology with SHIP, especially in the phosphatase and C terminal domains, as all three molecules can break down PIP₃ as well as interact with adapter molecules (March, 2002).

1.9 The SHIP Knockout Mouse

The biological importance of SHIP and the elucidation of its many functions have been exemplified in the SHIP knockout mouse (Helgason, 1998). The *ship*^{-/-} mouse was developed in 1998 by deleting the first exon of the SHIP gene, located on mouse chromosome 1c5, in the 129/SvJ mouse strain and has subsequently been back-crossed into a C57BL/6 background. The SHIP deletion has no effect on the expression of SHIP2, however sSHIP expression is elevated and more prolonged (Helgason, 1998). Overall, these mice are sickly; they suffer from various maladies including severe pulmonary inflammation, overproduction of granulocytes and macrophages, splenomegaly, extramedullary hematopoiesis, aberrant NK cell development, allograft rejection and osteoporosis (Helgason, 1998; Takeshita, 2002; Wang, 2002). Interestingly, these mice show similar phenotypes to others that have non-functional immune modulators, such as PTEN, SHP-1 and LYN. For example, all share increased proliferation of myeloid and monocytic cells, stem cell proliferation and hyper-responsivity to granulocyte/monocyte colony stimulating factor (GM-CSF) (Baran, 2003; Harder *et al.*, 2004; Neel *et al.*, 2003; Sly *et al.*, 2007; Xiao *et al.*, 2008b). These

phenotypes underscore the importance of regulating PIP₃ and its downstream signaling, by a variety of mechanisms.

1.10 The Function of SHIP in Innate Immune Cells

Examination of the function of SHIP in the *ship*^{-/-} mouse has uncovered the pluripotent activity of this molecule in almost every cell type of the immune response (Table 3). These effects are most profound in innate immune cells, where SHIP sets the activation threshold and controls proliferation in response to a variety of stimuli (March, 2002). For example, SHIP has been shown to act as a "gatekeeper" of mast cell degranulation. By negatively regulating PIP₃ levels in response to antigen and IgE, calcium flux is restricted and degranulation is modulated (Huber, 1998). SHIP also seems to control the IFN γ response of NK cells, as CD56 bright NK cells from human blood show markedly decreased levels of SHIP and produce more IFN γ upon activation (Trotta, 2004).

The function of SHIP in innate immune cells is best characterized in macrophages and monocytes. Because these cells derived from *ship*^{-/-} mice are hyper-responsive to cytokines and growth factors such as IL-3 and GM-CSF, (Kim, 1999) and the mice suffer from severe myeloproliferation (Helgason, 1998), it is clear that SHIPs control over macrophage activation and proliferation is essential in maintaining immune homeostasis. One of the major roles for SHIP in macrophages is to maintain tolerance to LPS. Endotoxin tolerance occurs when macrophages stimulated with low doses of LPS become transiently refractory to high LPS exposure, resulting in a dampened pro-inflammatory response initiated by the cell. This tolerance is thought to protect the host from activated immune cells as well as serve as a protective mechanism from persistent bacterial infections (West, 2002). Interestingly, *ship*^{-/-} macrophages do not display endotoxin tolerance and hypersecrete pro-inflammatory mediators such as TNF α , IL-6, IL-1 β and NO in response to LPS (Rauh, 2004; Sly, 2004).

In addition, SHIP plays an essential role in regulating macrophage phenotype. There are two general categories of macrophages in the immune system, M1 and M2. M1 macrophages are those effector cells we typically

associate with an anti-bacterial immune response; they produce ROS/RNS and cytokines central to controlling infection. In contrast, M2 macrophages produce much lower levels of inflammatory mediators, such as nitric oxide (NO), $\text{TNF}\alpha$, IL-12p70 and IL-23 after stimulation by pattern recognition receptor ligation and thus are ineffective in combating pathogens (Gordon and Taylor, 2005; Mantovani *et al.*, 2007). For example, M2 macrophages are impaired in their ability to limit the growth of intracellular *Mycobacterium tuberculosis* due to reduced NO production and increased iron levels within the phagosome, and they are also associated with murine susceptibility to cutaneous Leishmaniasis (Holscher *et al.*, 2006; Kahnert *et al.*, 2006). However, M2 macrophages do play a vital role in the resolution of immune responses and are essential for promoting tissue healing and repair (Gordon and Taylor, 2005). In macrophages, SHIP represses the generation of an M2 phenotype, presumably by limiting activation signals generated by PIP_3 (Rauh, 2005). Thus, SHIP is intimately involved in maintaining the delicate balance of macrophage differentiation, maturation and phenotype that ultimately has a dramatic effect on ensuring an appropriate response by the immune system.

Table 3. Regulation of immune cell functions by SHIP

Cell Type	SHIP Function	Reference
B Lymphocyte	↓ Proliferation ↓ Chemotaxis ↓ Activation	(Brauweiler <i>et al.</i> , 2000a)
T Lymphocyte	↓ Activation ↑ Apoptosis Th1/Th2 Bias	(Dong <i>et al.</i> , 2006; Tarasenko <i>et al.</i> , 2007)
Mast	↓ Degranulation ↓ Cytokine production ↓ Adherence	(Huber, 2000; Kalesnikoff <i>et al.</i> , 2002; Lam <i>et al.</i> , 2003)
Neutrophils	↓ Proliferation ↓ TLR2 Activation ↑ Survival	(Gardai, 2002; Hunter <i>et al.</i> , 2004; Strassheim <i>et al.</i> , 2005)
Platelets	↓ Activation ↓ Spreading	(Giuriato <i>et al.</i> , 2003)
NK	↓ IFN γ production	(Trotta, 2004)
DC	↓ Activation ↓ Progenitor number	(Neill <i>et al.</i> , 2007)
M Φ	Slows differentiation ↓ Proliferation ↑ Survival ↓ H ₂ O ₂ generation ↓ NF- κ B Signaling Skews phenotype to M2 ↓ TLR mediated LPS response ↓ Anti-bacterial activity	(Parsa, 2006; Sly <i>et al.</i> , 2007)

1.11 The potential role of SHIP during immune responses to *Salmonella*

Studies utilizing the *ship*^{-/-} mouse have made it clear that SHIP is an indispensable regulator of immune homeostasis, however, it is unknown how SHIP functions during an immune response following infection *in vivo*. Recent studies suggest that SHIP may direct the outcome of pathogenesis. For example, *in vitro*, SHIP regulates both the macrophage pro-inflammatory response to the intracellular pathogen, *Francisella novicida* (Parsa *et al.*, 2006), and phagosome maturation (Kamen *et al.*, 2007). This data correlates nicely with previously published work that has shown how other key regulators of immunity, like SHP-1,

are central to the outcome of disease (Neel *et al.*, 2003). In addition, SHIP is essential for maintaining endotoxin tolerance in both mice and macrophages (Sly *et al.*, 2004). Because many bacterial pathogens initially stimulate innate immune cells like macrophages through LPS and an appropriate inflammatory program is required to control infections, these studies suggest that an aberrant immune response may be mounted against such invaders in *ship*^{-/-} mice (Ohl and Miller, 2001). In addition, changes in SHIP expression in humans is correlated with various types of inflammatory disorders, such as severe allergies and peritonitis as well as cancers, suggesting that even in the absence of infection, dysregulation of SHIP is associated with disease (Jiang *et al.*, 2003; MacDonald and Vonakis, 2002; Muthukuru and Cutler, 2006).

1.12 Rationale

Complex immune responses are required to control *Salmonella* infections and SHIP is an essential regulator in many cell types, such as macrophages, that are central to preventing disease. Furthermore, SHIP dysfunction is associated with human diseases as well as impaired responses to pathogens *in vitro*. Based on this evidence, the central hypothesis examined in this thesis has been that SHIP is required to control *Salmonella* infection *in vivo*. In order to test this hypothesis, susceptibility to typhoid infection in *ship*^{-/-} mice and the innate immune mechanisms that may be affected in this model were explored. Because SHIP plays important roles in regulating macrophage behavior, and macrophages are central to both innate and adaptive/innate responses to *Salmonella*, it was further hypothesized that SHIP deficiency would impact the macrophage-dependent cytokine response to *Salmonella* infection, both *in vivo* and *in vitro*. This was tested by monitoring cytokine responses in mice during *Salmonella* infection (Chapter 3), and of infected BMDM (Chapter 4). During these studies it became clear that the role of SHIP in macrophage heterogeneity could be a defining aspect of the *ship*^{-/-} mouse's ability to resist *Salmonella* infection.

Throughout these studies of typhoid in the *ship*^{-/-} mouse, an interesting phenotype was observed in the gut that led to the data presented in chapter 5. Unless pretreated with streptomycin, mice do not get intestinal inflammation after infection with *S. Typhimurium*. However, this was not the case in *ship*^{-/-} mice, as they presented with severe inflammation of the ileum after oral infection with *Salmonella*. This led to the hypothesis that SHIP is required to modulate gut inflammatory responses during enteric infections. By examining gut histopathology and inflammatory mediator production in response to *Salmonella* and *Citrobacter rodentium* as well as heat-killed bacteria and LPS, it was found that SHIP deficiency predisposes mice to severe inflammation of the ileum in response to infection.

CHAPTER 2: MATERIALS AND METHODS

2.1 Bacterial culture and preparation

2.1.1 Growth conditions

S. Typhimurium SL1344 and *Citrobacter rodentium* DBS100 were grown overnight in 3 or 5 ml of Luria-Bertani broth (LB) respectively, at 37°C with shaking. To enumerate bacteria from overnight cultures, serial dilutions were prepared in sterile phosphate buffered saline ((PBS^{+/+}) + 0.901mM CaCl₂, HyClone, Mississauga, ON), plated on LB agar supplemented with 100ug/ml streptomycin (Gibco, Burlington, ON) and incubated for 24 hours at 37°C. Approximately 3x10⁹ bacteria were present in each 3 ml overnight culture.

2.1.2 Opsonization

For *in vitro* infections, *S. Typhimurium* SL1344 were opsonized by washing 100 µl of overnight culture and resuspending in 100 µl of 30% mouse serum in Dulbecco's Modified Eagle Media (DMEM, Hyclone) followed by incubation at 37°C for 25 min. Opsonized bacteria were then diluted 1:10 for infection of bone marrow-derived macrophages (BMDMs).

2.1.3 Bacterial killing

To heat-kill bacteria, 100 µl of overnight culture of *S. Typhimurium* SL1344 were washed and resuspended in 100 µl PBS^{+/+} followed by incubation at 80°C for 30 min. No heat-killed bacteria were viable after 48 hours incubation on LB agar at 37°C.

2.1.4 Preparation for infection

Prior to inoculation, heat-killed bacteria were centrifuged at 8,000 rpm for 4 min in an Eppendorf Mini Spin benchtop centrifuge (F45-12-11 rotor) and diluted 1:10 in 1 ml DMEM for infection of BMDMs.

2.2 *In vivo* infections

2.2.1 Mice

Six to eight week-old 129/SvJ x C57BL/6 F2 *ship*^{+/+} and *ship*^{-/-} mice were obtained from the laboratory of Dr. Gerald Krystal at the British Columbia Cancer Research Centre and bred for homozygotes. For mouse genotyping, the primers for SHIP A (sense oligo) 5'-TCTGTGCAGCTCAGTTTCCTCT-3', SHIP B (anti-sense oligo) 5'-CGTCCCACCATCCTATGACATAA-3' and TK Promotor (anti-sense oligo) 5'-CTGCATCTGCGTGTTCTGAATT-3' were obtained from Sigma Genosys (Oakville, ON, Canada). All *in vivo* experiments were performed in accordance with the protocols and guidelines provided by the Animal Care Committee at the University of British Columbia.

2.2.2 *Salmonella* Typhimurium infections

Mice were infected orally by gavage with a dose of 1×10^6 live *S. Typhimurium* SL1344, or intraperitoneally (IP) with 1×10^2 *S. Typhimurium* SL1344 for survival, bacterial load enumeration, cytokine analysis and immunohistochemistry experiments. For heat-killed survival experiments, mice were inoculated either orally by gavage or IP with 1×10^8 heat-killed *S. Typhimurium* SL1344. Oral inocula were diluted from overnight cultures in sterile HEPES buffer, pH 8.0 (Gibco), and IP inocula were diluted in sterile Hanks Balanced Salt Solution (HBSS, Sigma, Oakville, ON). Mice were sacrificed immediately when moribund (for survival experiments) and at two or five days post-infection (for bacterial load determination and cytokine analyses).

2.2.3 *Citrobacter rodentium* infections

Mice were infected orally by gavage with 1×10^8 *C. rodentium* DBS100, and sacrificed at two or 7 days post-infection for immunohistochemistry or bacterial load determination experiments, respectively. Oral inocula were diluted from overnight cultures in sterile HEPES buffer, pH 8.0 (Gibco).

2.3 Enumeration of bacterial load from infected mice

Mice were sacrificed two or five days post-infection for oral or IP *S. Typhimurium* infections, respectively, and colon, small intestine, liver, MLN and spleens were harvested into 1 ml of sterile, cold, PBS^{+/+}. For *C. rodentium* infections, mice were sacrificed 7 days post-infection and colons were harvested into 1 ml of sterile, cold, PBS^{+/+}. Organs were homogenized in 2ml Eppendorf Saftey-Lock tubes with one 7mm tungsten carbide bead using a Mixer Mill MM 200 (Retsch Technologies, Haan, Germany) and serial dilutions of homogenate were prepared in sterile PBS^{+/+} for plating. Bacteria were grown on LB Agar + 100µg/ml streptomycin for 24 hours at 37°C and subsequently counted.

2.4 *In vivo* cytokine analyses

For analysis of mouse serum cytokine levels, blood was obtained by cardiac puncture at various time points post-infection (as indicated in legends), incubated at 37°C for 1 hour and then spun at 13,200 rpm in an Eppendorf 5415D benchtop centrifuge for 10 min to separate serum. Serum was aliquoted and frozen at – 80°C and analyzed using the mouse inflammation cytometric bead array (CBA) assay kit (BD Biosciences, Mississauga, ON) as per manufacturer's instructions. Briefly, cytokine levels were analyzed in a multiplex fashion, whereby single samples of mouse serum were incubated with a combination of antibody coated beads specific for IL-12p70, IL-6, IL-10, TNF α and IFN γ and phycoerythrin (PE) detector solution. Samples were assessed by flow cytometry on a BD FacsCalibur Flow Cytometer and cytokine levels then analyzed using the kit associated CBA software (BD Biosciences).

Table 4. Antibodies and dyes used in studies. (WB) Western blot, (IHC) Immunohistochemistry, (FACS) Fluorescence activated cell sorting.

Antibody	Species	Dilution	Use	Source
YM-1	rabbit	1:10,000, 1:500	WB, IHC	Stem Cell Technology, Vancouver, BC
SHIP	mouse	1:10,000	WB	Santa Cruz Biotechnology, Santa Cruz, CA
Arg-1	mouse	1:10,000, 1:100	WB, IHC	BD Transduction, Lexington, KY
GAPDH	mouse	1:10,000	WB	Research Diagnostics, Flanders, NJ
Biotin F/480	rat	1:1000, 1:100	IHC, FACS	ADB Serotec, Hornby, ON
Biotin GR-1	rat	1:100	FACS	BD Biosciences, Mississauga, ON
apc Mac-1	rat	1:100	FACS	BD Biosciences, Mississauga, ON
PE CD11c	hamster	1:200	FACS	BD Biosciences, Mississauga, ON
PE CD-3	hamster	1:400	FACS	BD Biosciences, Mississauga, ON
Biotin B220	rat	1:200	FACS	BD Biosciences, Mississauga, ON
Mouse-HRP	goat	1:5000	WB	Invitrogen, Burlington, ON
Rabbit-HRP	goat	1:5000	WB	Invitrogen, Burlington, ON
Rat -568 AlexaFluor®	goat	1:200	IHC	Invitrogen, Burlington, ON
Rabbit-488 AlexaFluor®	goat	1:200	IHC	Invitrogen, Burlington, ON
Mouse-468 AlexaFluor®	goat		IHC	Invitrogen, Burlington, ON
SA-FITC	-	1:400	FACS	BD Biosciences, Mississauga, ON
7AAD	-	1:250	FACS	BD Biosciences, Mississauga, ON

2.5 Immunohistochemistry

2.5.1 Preparation of tissues

Tissues were removed from mice and immediately fixed in 10% neutral buffered formalin and incubated at 23°C for 24 hours and then transferred into 70%

ethanol. Fixed tissues were embedded in paraffin, cut into 5mm sections and either left untreated, or stained for hematoxylin and eosin (H&E) and Masson's trichrome stain using standard techniques by Wax-it Histology Services (Vancouver, BC, Canada).

2.5.2 Immunohistochemistry for M2 macrophages

For immunohistochemistry staining, slides with 5mm sections of tissue were de-paraffinized and re-hydrated. Antigen retrieval was carried out by digesting tissues in 20mg/ml proteinase K (Sigma) in TE buffer (50mM Tris base, 1mM EDTA, pH 8.0) for 15 min. Tissues were then immunostained with primary antibodies against Ym-1, arginase 1 (Arg1) and F4/80, followed by incubation with secondary antibodies. After staining, cover slips were mounted over tissues using ProLong Gold Antifade reagent (Invitrogen) containing DAPI for DNA staining. Images were obtained using a Zeiss AxioImager microscope equipped with and AxioCam HRm camera operating through AxioVision software (Carl Zeiss Ltd., Toronto, ON, Canada).

2.5.3 Pathology Scoring

Tissue sections prepared in H&E stain by Waxit Histology Services were scored for inflammation in the lumen, surface epithelium, mucosa and submucosa using the criteria outlined in appendix 5.

2.6 Western Analysis

2.6.1 *Ex-vivo* cell preparation

Peritoneal macrophages were obtained from infected and uninfected mice by lavage with 10 ml DMEM (HyClone) + 10% FBS (Gibco). Cells were washed 1 time in DMEM, counted, spun at 1000 rpm in a Beckman Coulter Allegra X-12 R benchtop centrifuge at 23°C for 5 min and lysed directly into 1× Laemmli's Western sample buffer.

2.6.2 SDS-PAGE and Western blotting

Samples were boiled for 3 min in Laemmli's sample buffer and spun at 13,200 rpm in an Eppendorf 5415D benchtop centrifuge for 30 sec before loading on 10% SDS-polyacrylamide gels for electrophoresis. Proteins were transferred onto nitrocellulose membranes (Millipore, Bedford, MA) using a semi-dry transfer system (BioRad, Mississauga, ON). The membranes were incubated for one hour at 23°C in tris-buffered saline + 0.1% Tween 20 (TBST) + 5% FBS (blocking buffer) and then incubated overnight at 4°C with primary antibodies against Ym1, Arg1, SHIP or GAPDH diluted in blocking buffer. After washing 3 x 10 min in TBST, membranes were incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies diluted in blocking buffer for 1 hour at 23°C. Membranes were washed again 3 x 10 min in TBST and proteins were detected using a chemiluminescence (ECL) kit according to manufacturer's instructions (Amersham Biosciences, Piscataway, NJ).

2.7 Flow Cytometry

2.7.1 Leukocyte isolation

Spleens and MLN from orally infected mice were pooled and harvested into 5 ml DMEM + 1% HEPES Buffer + 0.01mg/ml *V. alginolyticus* collagenase (Roche Diagnostics, Indianapolis, IN) and then minced using scissors and forceps, followed by incubation at 37°C for 1 hr. Cell suspensions were separated by drawing through an 18 gauge needle 3 times with a 5 ml syringe. Cells were washed in PBS^{-/-} (without added calcium) + 2% FBS + 0.5% NaN₃ (FACS buffer), centrifuged at 1200 rpm in a Beckman Coulter Allegra X-12 R benchtop centrifuge at 23°C for 5 min and resuspended in 10 ml FACS buffer for flow cytometry staining.

2.7.2 Leukocyte staining for flow cytometry

Approximately 1x10⁶ cells were stained per well in 96 well round bottom tissue culture plates with primary antibodies against CD3, B220, F4/80, Mac-1, Gr-1

and CD11c diluted in FACS buffer for 30 min at 4°C. Cells were then washed 2 x in FACS buffer, followed by secondary conjugate staining for 30 min at 4°C. Cells were washed again 2 times and resuspended in 300µl FACS buffer and transferred into FACS tubes for acquisition. Percent positive cells were assessed using a Cell Quest Pro software on a BD Facscalibur Flow Cytometer and analyzed using FlowJo flow cytometry software (Tree Star, Ashland, OR).

2.8 Macrophage Tissue Culture

2.8.1 BMDMs

Bone marrow cells were obtained by flushing tibiae and femora from uninfected *ship*^{+/+} and *ship*^{-/-} mice. 5x10⁶ cells were first suspended in 10 ml DMEM supplemented with 10% heat-inactivated FBS, 2mM L-glutamine, 1mM sodium pyruvate, 100U/ml penicillin/100µg/ml streptomycin (Gibco) and 30% conditioned media from L929 cells as a source of macrophage colony stimulating factor (MCSF). M2 inducing media was further supplemented with 2% mouse serum taken from *ship*^{+/-} mice. After allowing cells to adhere to non-tissue culture treated petri plates for 3 hours at 37°C and 5% CO₂, non-adherent cells were transferred to fresh petri plates for differentiation at 37°C, 5% CO₂ for 10 days with complete media changes on adherent cells at days four and 7. This procedure results in a population of cells that is 95% positive for the macrophage markers F4/80 and Mac-1 (Sly *et al.*, 2004).

2.8.2 Raw 264.7 macrophages

Raw 264.7 macrophages were obtained from American Type Culture Collection cell biology stock centre (Rockville, MD). Cells were grown in culture in DMEM + 10% FBS and skewed to an M2 phenotype 24 hours prior to infection using 100ng/ml recombinant mouse IL-4 (R&D Systems, Minneapolis, MN).

2.9 *In vitro* infections

2.9.1 Preparation of BMDMs

Cells were removed from petri plates using non-enzymatic cell dissociation buffer (Gibco) and washed twice in 10 ml DMEM to remove antibiotics. Cells were seeded at a density of 1×10^5 cells/well in 1 ml of DMEM + 10% FBS, 2mM L-glutamine and 1mM sodium pyruvate (BMDM media).

2.9.2 Preparation of Raw 264.7 macrophages

Cells were removed from petri plates using non-enzymatic cell dissociation buffer and washed twice in 10 ml DMEM to remove antibiotics. Cells were seeded at a density of 1×10^5 cells/well in 1 ml of DMEM + 10% FBS + 100ng/ml recombinant mouse IL-4 (raw media, R&D Systems) in 24 well tissue culture plates 12 hours prior to infection.

2.9.3 *S. Typhimurium* infections

Cells were infected at a multiplicity of infection of 10 with either opsonized *S. Typhimurium* SL1344 or inoculated with heat-killed *S. Typhimurium* SL1344 and centrifuged at 1,000rpm in a Beckman GS-6R benchtop centrifuge at 23°C for 5 min to synchronize infection, followed by incubation at 37°C, 5% CO₂ for 15 min. To remove extracellular bacteria, cells were washed three times in sterile PBS^{+/+} and supplied with 1 ml BMDM or raw media + 50µg/ml gentamicin (Sigma) for 2 hours. Cell supernatants then were replaced with 1 ml BMDM or raw media + 10µg/ml gentamicin. LPS-treated control cells were given BMDM media + gentamicin + 100ng/ml *S. Typhimurium* LPS (Sigma) for the amounts of time indicated.

2.10 Intracellular replication (gentamicin protection) assays

BMDMs and Raw 264.7 macrophages were infected with *S. Typhimurium* SL1344 as above and supernatants were removed at two and 24 hours post-infection. After washing 3 times in PBS^{+/+}, cells were lysed in 250 µl PBS^{+/+} + 1% TritonX-100 + 0.1% SDS. Lysates were serially diluted and plated on LB Agar + 100 µg/ml streptomycin for 24 hours at 37°C and subsequently counted. Fold replication numbers were generated by dividing the bacterial load enumerated from 24 hour lysates by 2 hour lysates.

2.11 Cell death assays

BMDMs were infected with live, or inoculated with heat-killed *S. Typhimurium* SL1344, or treated with LPS and supernatants were removed at 8 and 24 hours post-infection. After washing three times with PBS^{+/+}, cells were scraped using a rubber scraper from the wells into 300 µl FACS buffer. Cells were transferred to 96 well round bottom tissue culture plates and stained for necrosis using 7-amino-actinomycin D (7AAD). 7AAD positivity was assessed by flow cytometry using a Cell Quest Pro software on a BD FACS Calibur flow cytometer and samples were analyzed using FlowJo flow cytometry software (BD Biosciences).

2.12 Cytokine analyses

At designated time points post-infection, 1 ml of supernatant was removed from each well of infected or control BMDMs, aliquoted and frozen at -80°C until assayed for cytokines using mouse TNF α , IL-10, IL-6 and IL-12p70 ELISA kits (BD Biosciences).

2.13 Collagen assays

Approximately 0.5 cm or 0.10 g of ilea was removed from mice at two days post-oral infection and immediately placed into 500 µl of 0.5M Acetic Acid + 10mg/ml pepsin (Sigma). Samples were incubated overnight at 23°C with rotation followed by the addition of 1 ml Sircol dye (Biocolor, N. Ireland). Samples were incubated for 1 hour at 23°C and spun for 10 minutes at 10,000 rpm in an Eppendorf 5415D benchtop centrifuge followed by the removal of supernatant. Bound dye was

resuspended in 1 ml 0.5 M Alkali Solution (Biocolor) and incubated at 23°C for 10 min. Samples were vortexed and 100 µl were transferred to a 96 well clear bottom microplate. Fluorescence was read with a TECAN SpectraFluor Plus plate reader (TECAN, Research Triangle Park, NC) at wavelength of 560nm.

2.14 Statistical analyses

For *in vivo* time of death experiments, logrank statistical analyses for survival data were performed on curves generated in Graph Pad Prism 4.0 (MacKiev Software). For enumeration of bacterial loads from infected mice as well as replication assay, flow cytometry, ELISA and CBA data, statistical analyses were performed using two-tailed, unpaired student's *t* tests with a 95% confidence interval in Graph Pad Prism 4.0 (MacKiev Software). For lymphocyte distributions, one-way analysis of variance (ANOVA) with a 95% confidence interval were performed with Bonferroni tests applied post-hoc. On all graphs, error bars represent standard error from the arithmetic mean. Statistical significance based on P values of <0.05, <0.01 and <0.001 are represented on graphs by one, two or three asterisks, respectively.

CHAPTER 3: The role of SHIP in *S. Typhimurium* infections *in vivo*

3.1 Introduction

SHIP negatively regulates various hematopoietic cell functions and is critical for balancing pro- and anti-inflammatory responses of both innate and adaptive cells. Negative regulation is particularly important during bacterial infections in order to create the proper interface between innate and adaptive immunity and to protect the host from potent inflammatory mediators. This is especially true during infections with pathogens like *Salmonella*, where strong innate responses are required for an extended period of time in order to prevent bacterial replication. Because alterations in SHIP function have been implicated in various human diseases as well as interactions with pathogens in cell culture, it is important to examine the role it may play *in vivo* during the pathogenesis of *Salmonella*, which has such an impact on global health.

To test the hypothesis that SHIP is required to control *Salmonella* infection *in vivo*, susceptibility and the innate immune responses of *ship*^{+/+} and *ship*^{-/-} mice to *S. enterica* serovar Typhimurium infection were compared. Survival experiments and enumeration of bacterial loads in intestinal and systemic sites indicated the high degree of susceptibility to *Salmonella* infections in *ship*^{-/-} mice. Cytokine analysis of mouse serum-used to profile innate immune responses in the presence or absence of SHIP showed that *ship*^{-/-} mice produce lower levels of Th1 polarizing cytokines compared to *ship*^{+/+} animals at two days post-infection, and time courses revealed the defective nature of the cytokine response throughout the duration of infection. To investigate the possibility that M2 macrophages could be contributing to this cytokine profile, these cells were looked for at sites of *Salmonella* infection. Indeed, M2 macrophages were the predominant population *in vivo*; tissue macrophages within the small intestine and peritoneal macrophages from *ship*^{-/-} mice showed elevated levels of the M2 macrophage markers Ym1 and Arg1 compared to *ship*^{+/+} cells. When the distribution of other lymphocyte populations was examined, it was found that in correlation with previously published studies, there are inherent differences between macrophage and B cell populations between *ship*^{+/+} and *ship*^{-/-} mice, however these differences were not exaggerated upon infection. These results

indicate that SHIP does indeed play a crucial role in modulating the immune response during *Salmonella* pathogenesis *in vivo* and suggests a contribution of the M2 macrophage in this system.

3.2 Results

3.2.1 SHIP controls susceptibility to *Salmonella* infection *in vivo*

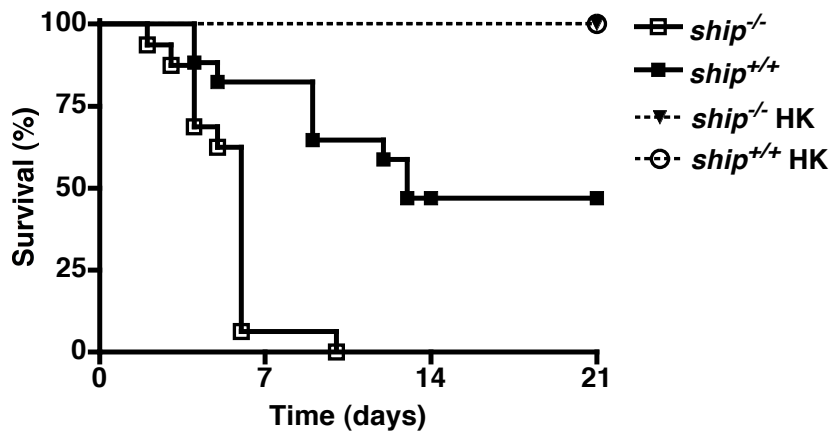
Because endotoxin tolerance is associated with resistance to *Salmonella* infection (Lehner *et al.*, 2001) and this response is defective in *ship*^{-/-} mice (Sly *et al.*, 2004), it was hypothesized that these animals would be more susceptible to Gram-negative bacterial infection. To test this, *ship*^{+/+} and *ship*^{-/-} mice were infected orally or IP with *S. Typhimurium* and their survival was monitored. *ship*^{-/-} mice were significantly more susceptible to oral *Salmonella* infection compared to *ship*^{+/+} mice (P<0.001, Fig. 5 A). Even with a low dose of 1x10⁶ bacteria, *ship*^{-/-} mice died as early as day two post-infection and no animals survived longer than 10 days, while 47% of *ship*^{+/+} mice survived to at least 21 days post-infection. This phenotype was not specific to oral *Salmonella* infection, since *ship*^{-/-} mice were significantly more susceptible to *Salmonella* following IP infection with 1x10² bacteria than *ship*^{+/+} mice (P=0.0119, Fig. 5 B).

Administration of LPS via IP injection is lethal to *ship*^{-/-} mice within 54 hours (Sly *et al.*, 2004). Therefore, the possibility that death seen at early time points after oral or IP infection in *ship*^{-/-} mice was due to endotoxic shock from *Salmonella* LPS in the infection inoculum was examined. To do this, *ship*^{+/+} and *ship*^{-/-} mice were inoculated orally or IP with a dose of 1x10⁶ or 1x10² heat-killed *Salmonella*, respectively, and survival was monitored. Results showed that 100% of both *ship*^{+/+} and *ship*^{-/-} mice survived these treatments (Fig. 5). In addition, 100% of mice infected either orally or IP with a high dose of 1x10⁸ heat-killed *Salmonella*, which more closely mimicked bacterial load levels seen when mice were moribund, survived infection (Figs. 6 and 7). Furthermore, *ship*^{-/-} mice were not susceptible to sepsis induced by LPS found on replicating *Citrobacter*

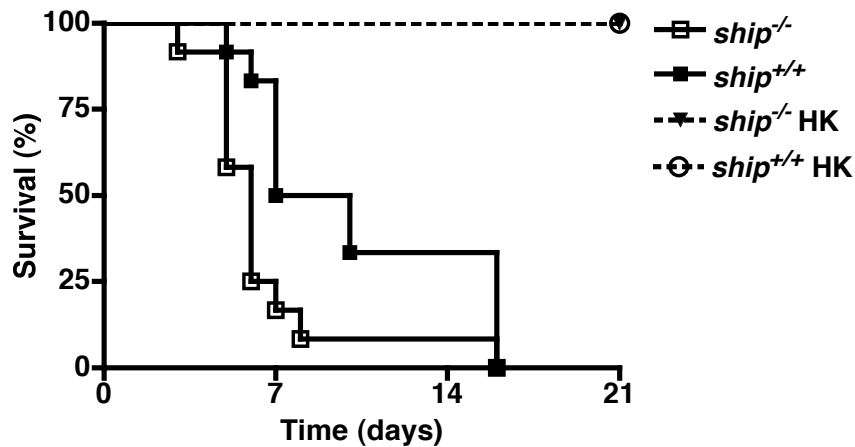
rodentium (Fig. 8). While the possibility that LPS may still play some role in mortality in *ship*^{-/-} mice cannot be completely excluded, these data suggest that while *ship*^{-/-} mice can not control *Salmonella* replication, levels of LPS present in infection inocula are not sufficient to cause mortality.

Figure 5. SHIP controls susceptibility to *Salmonella* in vivo. (A) *ship*^{+/+} and *ship*^{-/-} mice were infected orally with 1x10⁶ live, or 1x10⁶ and 1x10⁸ heat-killed (HK) *S. Typhimurium* SL13344 and time of death was assessed over 3 weeks. (B) *ship*^{+/+} and *ship*^{-/-} mice were infected IP with 1x10² live, or 1x10² and 1x10⁸ heat-killed (HK) *S. Typhimurium* SL13344 and time of death was assessed over 2 weeks. For A and B three independent experiments were performed with a total N=12 for both *ship*^{+/+} and *ship*^{-/-} mice.

A.



B.



3.2.2 Increased susceptibility is associated with high bacterial load in *ship*^{-/-} mice

Higher bacterial burdens in the organs of infected mice corresponded to increased susceptibility to infection. During oral *S. Typhimurium* infection, the most significant differences were seen on days two and four-post infection. On day two, bacterial loads were significantly higher in all organs except the small intestines (SI) of *ship*^{-/-} mice compared to *ship*^{+/+} mice (spleen P=0.0245, liver P=0.0459 and colon P=0.005) and significantly higher in all organs taken on day four (spleen P=0.0828, liver P=0.0238, SI P=0.0013 and colon P=0.0103) (Fig. 6). During IP infections, colony counts were also significantly higher in all organs taken from *ship*^{-/-} mice compared to *ship*^{+/+} mice at days two, three, and five post-infection, and all organs except liver, on day four post-infection (Table 5, Fig. 7).

Table 5. Statistical Significance of CFU values in *ship*^{-/-} vs. *ship*^{+/+} organs after IP *Salmonella* infection

	<i>ship</i> ^{+/+} Colon	<i>ship</i> ^{-/-} SI	<i>ship</i> ^{-/-} Spleen	<i>ship</i> ^{-/-} Liver
Day 2	P=0.0248	P=0.0085	P=0.0136	P=0.0122
Day 3	P=.0069	P=.0020	P=0.0007	P=0.0002
Day 4	P=0.0017	P=0.0040	P=0.0276	NS
Day 5	P=0.005	P=0.0011	P=0.0230	P=0.0068

Figure 6. Increased susceptibility to *Salmonella* is associated with higher bacterial loads in organs of orally infected mice. *ship*^{+/+} and *ship*^{-/-} mice were infected orally with 1x10⁶ *S. Typhimurium* SL1344 and sacrificed at days 1-4 days post-infection (A-D). Colon, SI, liver, and spleen were harvested from the mice, homogenized and plated to enumerate bacterial load. 3 independent experiments were performed with a total N=9 for days 1-3, or N=12 on day 4, for both *ship*^{+/+} and *ship*^{-/-} mice.

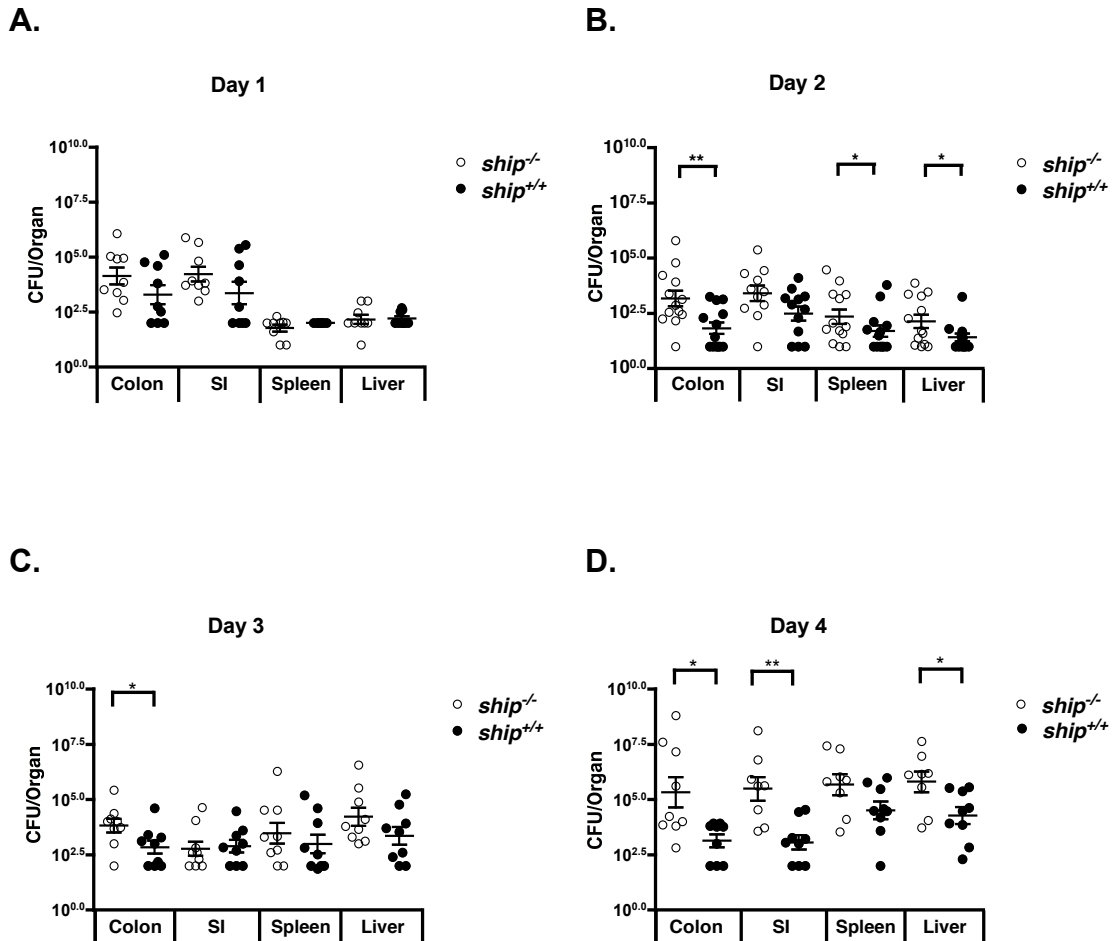
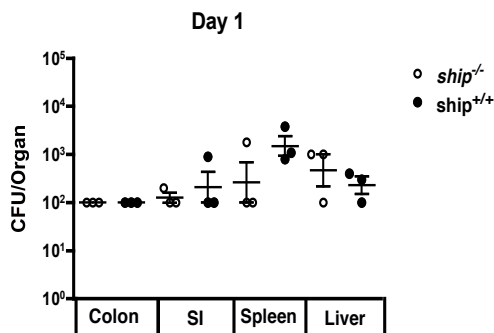
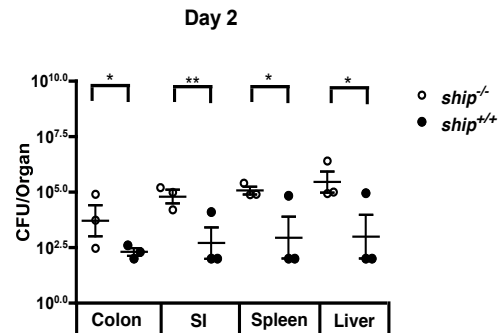


Figure 7. Increased susceptibility to *Salmonella* is associated with higher bacterial loads in organs of IP infected mice. *ship*^{+/+} and *ship*^{-/-} mice were infected IP with 1x10² *S. Typhimurium* SL13344 and sacrificed at 1-5 days post-infection (A-E). Colon, SI, liver and spleen were harvested from the mice, homogenized and plated to enumerate bacterial load. 3 independent experiments were performed with a total N=3 for days 1-4, or N=12 on day 5, for both *ship*^{+/+} and *ship*^{-/-} mice.

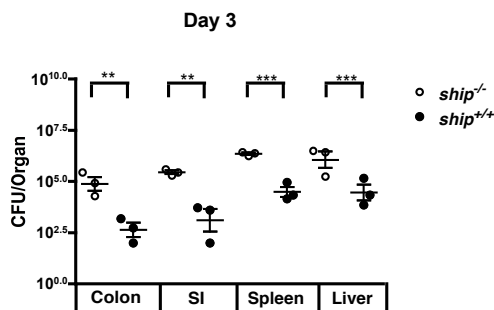
A.



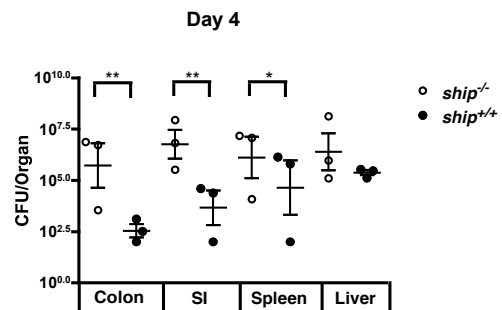
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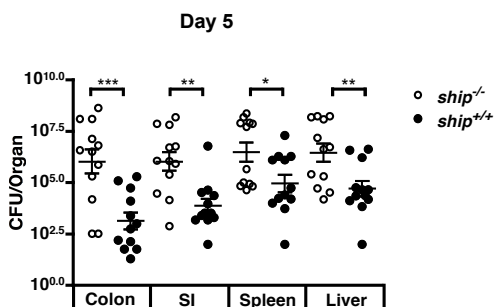
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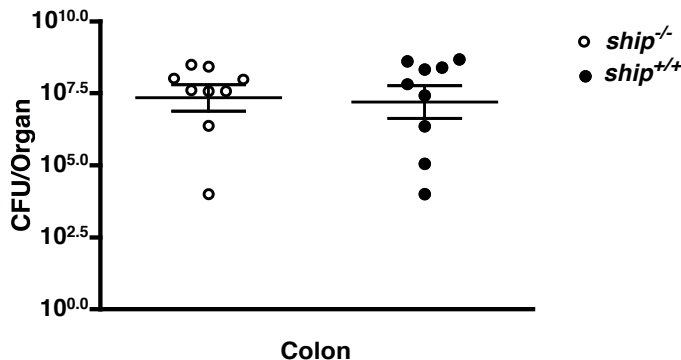


E.



Importantly, I found that when challenged with *Citrobacter rodentium*, an extracellular attaching and effacing pathogen that does not cause systemic disease (Mundy *et al.*, 2005), there was no difference in colonization of the colon between *ship*^{+/+} and *ship*^{-/-} mice (Fig. 8) and infection in either strain did not lead to morbidity or mortality in mice used in CFU experiments after 7 days. These data suggest that the outcome of infection in *ship*^{-/-} mice may be dependent on the intracellular or extracellular nature of the pathogen and it highlights the role SHIP may play in preventing systemic infection.

Figure 8. *ship*^{+/+} and *ship*^{-/-} mice are equally susceptible to colonization by *Citrobacter rodentium*. *ship*^{+/+} and *ship*^{-/-} mice were infected orally with 1x10⁸ *C. rodentium* DBS100 and sacrificed at 7 days post-infection. Colons were harvested from the mice, homogenized and plated to enumerate bacterial load. 3 independent experiments were performed with a total N=9 for both *ship*^{+/+} and *ship*^{-/-} mice.



3.2.3 *ship*^{-/-} mice produce levels of inflammatory cytokines typical of M2 macrophages during *Salmonella* infection

IL-12 and IFN γ comprise the central axis of Th1 polarizing cytokines that are known to drive the immune response against *Salmonella* (Eckmann and Kagnoff, 2001; Mastroeni, 2002). Thus, the association between increased susceptibility to *Salmonella* in *ship*^{-/-} mice and lower concentrations of these cytokines during infection was examined. Mice were infected orally or IP with 1x10⁶ or 1x10² *S. Typhimurium* SL1344, respectively, and blood was taken from

the mice at two days after oral infection or five days after IP infection for cytokine analyses. These were the same animals used to generate bacterial load data shown in figures 6 B and 7 E, and these days were chosen to prevent *ship*^{-/-} mice from becoming moribund during oral or IP infections. In response to oral infection, *ship*^{-/-} mice produced significantly lower amounts of IFN γ (P=0.0377), IL-6 (P=0.0259) and IL-10 (P=0.0301) than *ship*^{+/+} mice, and production of IL-12p70 was also decreased, albeit not significantly (Fig. 9 A). In an oral time course experiment where cytokine levels were examined on days one to four post-infection (Fig. 9 B-E) it was also observed that *ship*^{-/-} mice produce lower levels of IL-12p70, IFN γ , IL-10 and IL-6 in comparison to *ship*^{+/+} mice. During an IP infection, a similar trend was observed on day five post-infection as oral infection cytokines taken on day two post-infection, with the exception of IL-6; *ship*^{-/-} mice produced significantly higher levels of IL-6 (p=0.0299) but lower levels of IL-12p70 and IFN γ than *ship*^{+/+} mice, however these differences were not significant (Fig. 10 A). Tracking the kinetics of cytokine production during an IP infection showed that while *ship*^{-/-} mice produced lower levels of IL-12p70, IFN γ , IL-10 and IL-6 at early time points, by day three these levels were much higher than those produced in *ship*^{+/+} mice. In the case of IL-12p70, IFN γ and IL-10, production in *ship*^{-/-} mice was lower than *ship*^{+/+} at day five post-infection. In both oral and IP infections TNF α levels were significantly higher in *ship*^{-/-} than in *ship*^{+/+} mice (P=0.0173 and P=0.0085, respectively) throughout the duration of infection (Fig. 9 A and C, Fig. 10 A and C). Importantly, no significant differences were found between cytokine levels in uninfected *ship*^{-/-} and *ship*^{+/+} mice (Fig. 11) and altered cytokine levels in *ship*^{-/-} mice correlate with increased bacterial load in organs. Trends toward low IL-12 and IFN γ levels produced in *ship*^{-/-} mice upon *Salmonella* infection suggest a cytokine profile characteristic of M2 macrophages; therefore, association between increased susceptibility to *Salmonella* and SHIP deficiency could be due to a lack of M1 macrophages that are important for skewing of Th1 responses required to prevent disease.

Figure 9. SHIP deficiency leads to altered levels of inflammatory cytokine production after oral *Salmonella* infection *in vivo*. *ship*^{+/+} and *ship*^{-/-} mice were infected orally with 1x10⁶ *S. Typhimurium* SL1344 and sacrificed on days 1-4 post-infection. Blood samples were obtained via cardiac puncture and serum was separated for cytokine analysis. Cytokines were analyzed using the CBA mouse inflammation kit. (A) Cytokine production on day 2 post-infection. (B-F) Kinetics of cytokine production during a 4 day oral infection. 3 independent experiments were performed with a total N=9 for days 1-4, or N=12 on day 2, for both *ship*^{+/+} and *ship*^{-/-} mice.

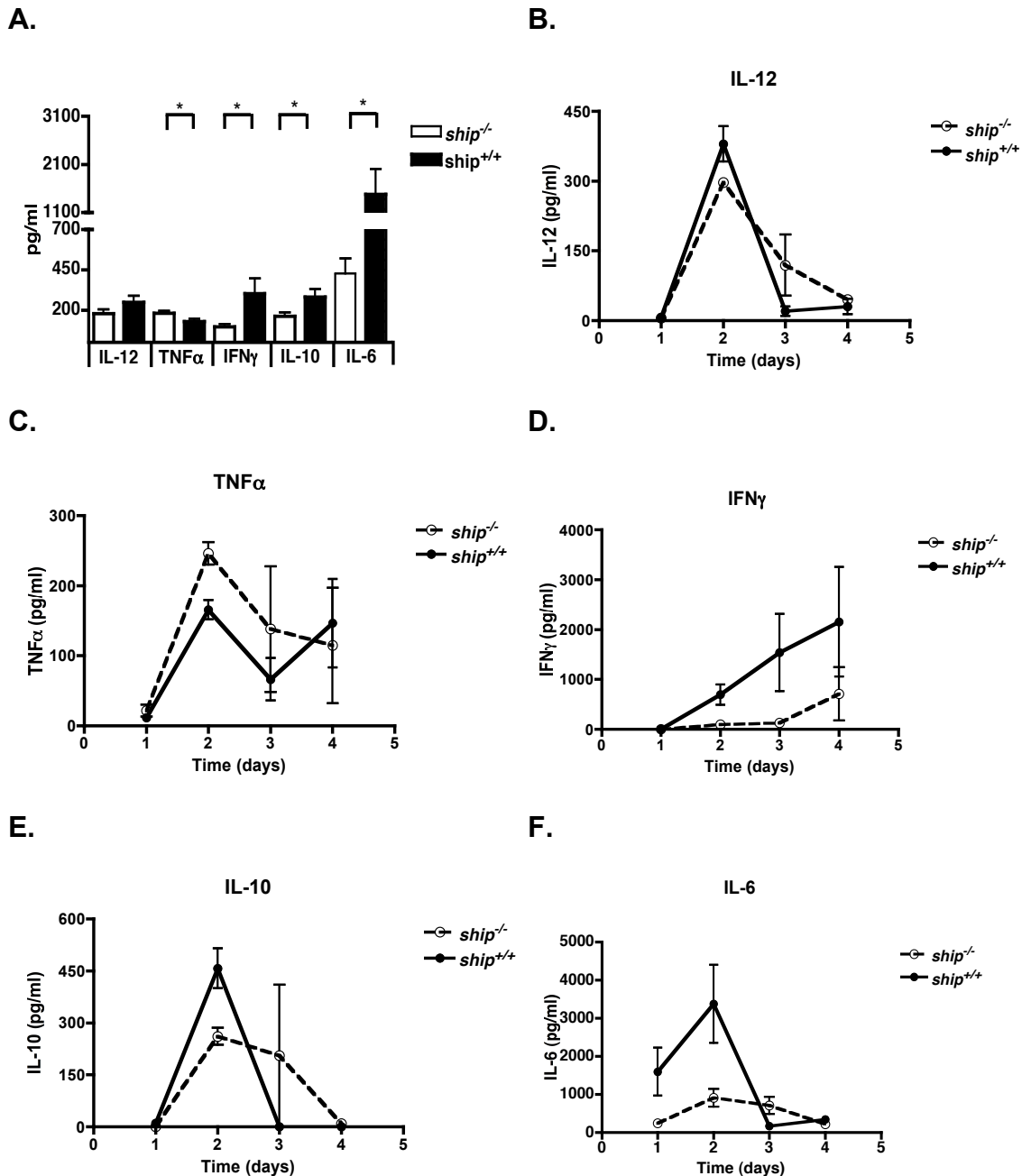


Figure 10. SHIP deficiency leads to altered levels of inflammatory cytokine production after IP *Salmonella* infection *in vivo*. *ship*^{+/+} and *ship*^{-/-} mice were infected IP with 1x10² *S. Typhimurium* SL1344 and sacrificed on days 1-5 post-infection. Blood samples were obtained via cardiac puncture and serum was separated for cytokine analysis. Cytokines were analyzed using the CBA mouse inflammation kit. (A) Cytokine production on day 5 post-infection. (B-F) Kinetics of cytokine production during a 5 day IP infection. 3 independent experiments were performed with a total N=9 for days 1-4, or N=12 on day 5, for both *ship*^{+/+} and *ship*^{-/-} mice.

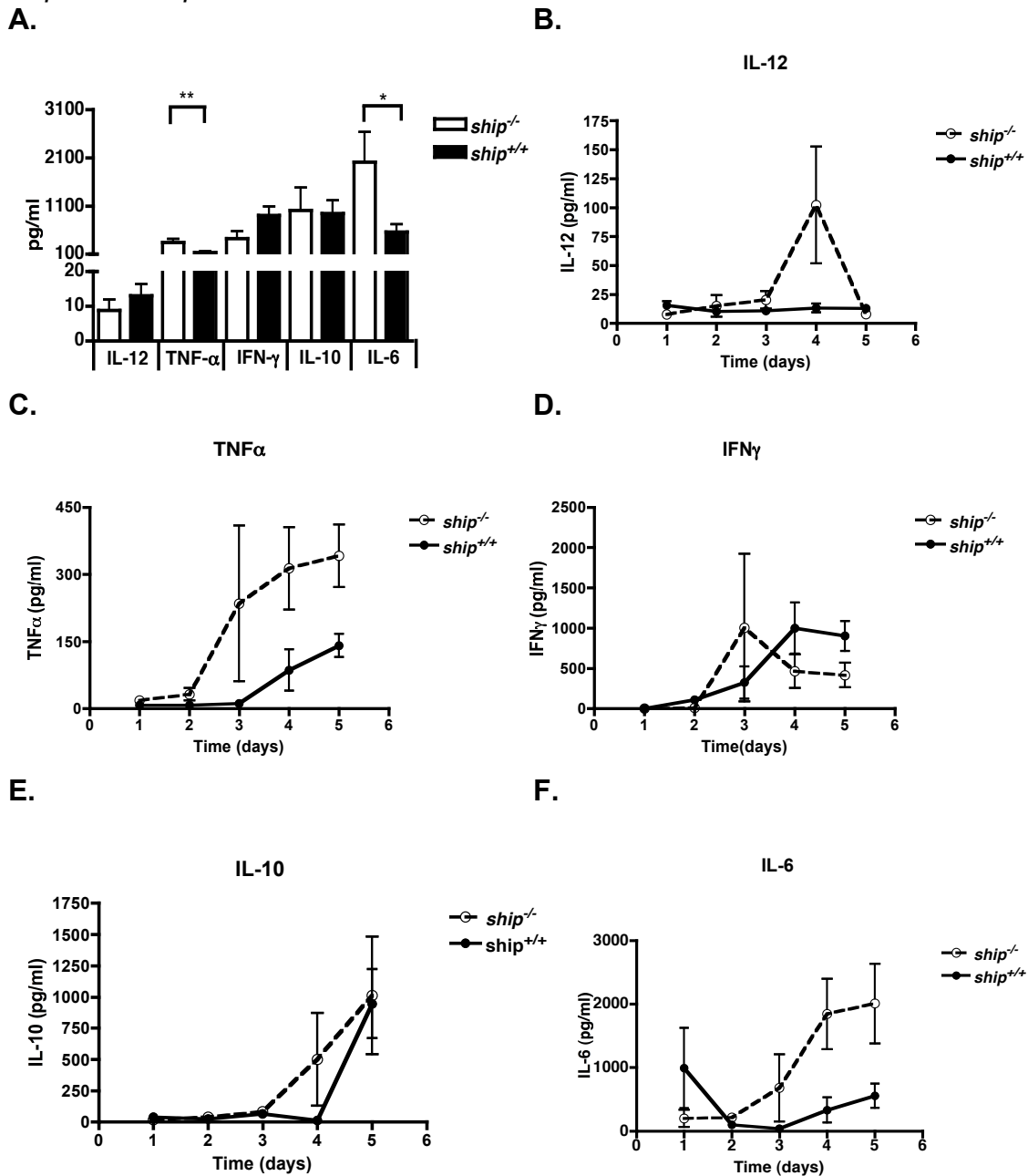
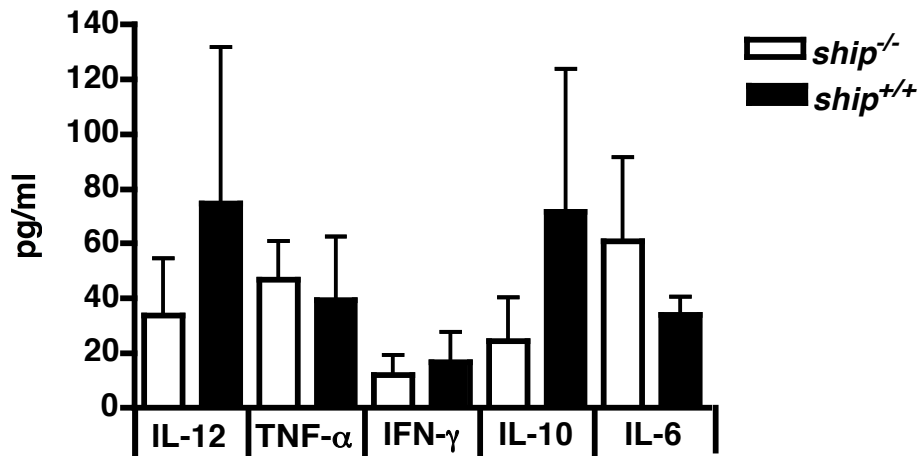


Figure 11. Cytokine production in uninfected *ship*^{-/-} and *ship*^{+/+} mice. Uninfected *ship*^{+/+} and *ship*^{-/-} mice were sacrificed, blood samples were obtained via cardiac puncture, and serum was separated for cytokine analysis. Cytokines were analyzed using the flow cytometry based CBA mouse inflammation kit. 3 independent experiments were performed with a total N=12 for both *ship*^{+/+} and *ship*^{-/-} mice.



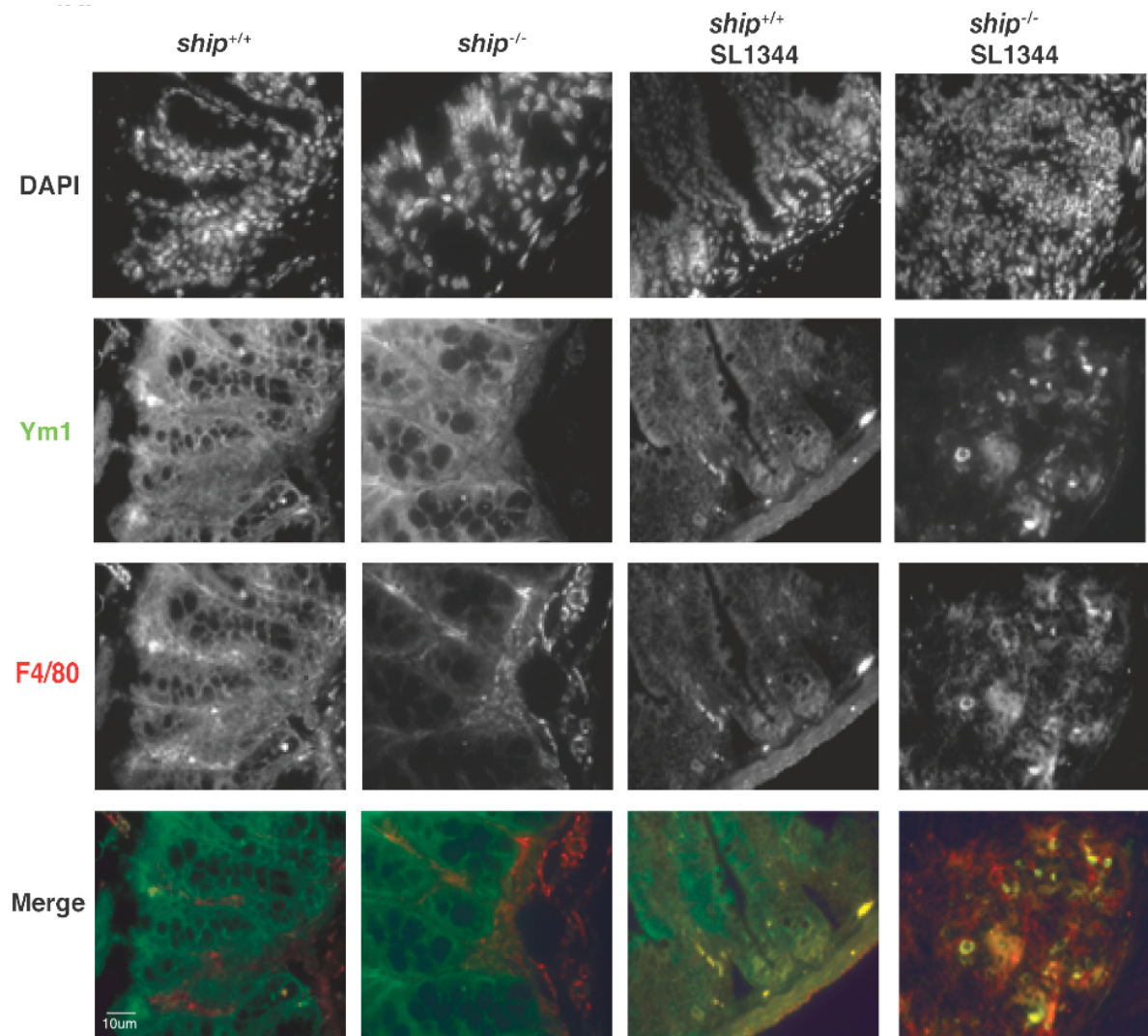
3.2.4 M2 macrophage skewing is associated with increased susceptibility to *Salmonella* in *ship*^{-/-} mice

Previous work has shown that SHIP deficiency skews the macrophage phenotype *in vivo* towards M2 in the lung and peritoneal cavity (Rauh *et al.*, 2005) and M2 macrophages are ineffective at mounting immune responses against pathogens, especially those requiring Th1 cytokines for clearance (Mantovani *et al.*, 2007; Parsa *et al.*, 2006). Therefore, it was hypothesized that increased susceptibility to *Salmonella* infection in *ship*^{-/-} mice could be due, in part, to a lack of M1 effector macrophages. To address whether macrophages in *ship*^{-/-} mice were M2, the presence of two M2 macrophage markers, Ym1 and Arg1, in tissue sections and peritoneal macrophages isolated from both uninfected mice and those infected orally or IP with *S. Typhimurium* SL1344, was assessed. Histological sections of the small intestine of orally infected *ship*^{-/-} mice showed a large amount of Ym1 and Arg1 positive macrophages in the submucosa, whereas infected *ship*^{+/+} mice showed few Ym1 or Arg1 positive

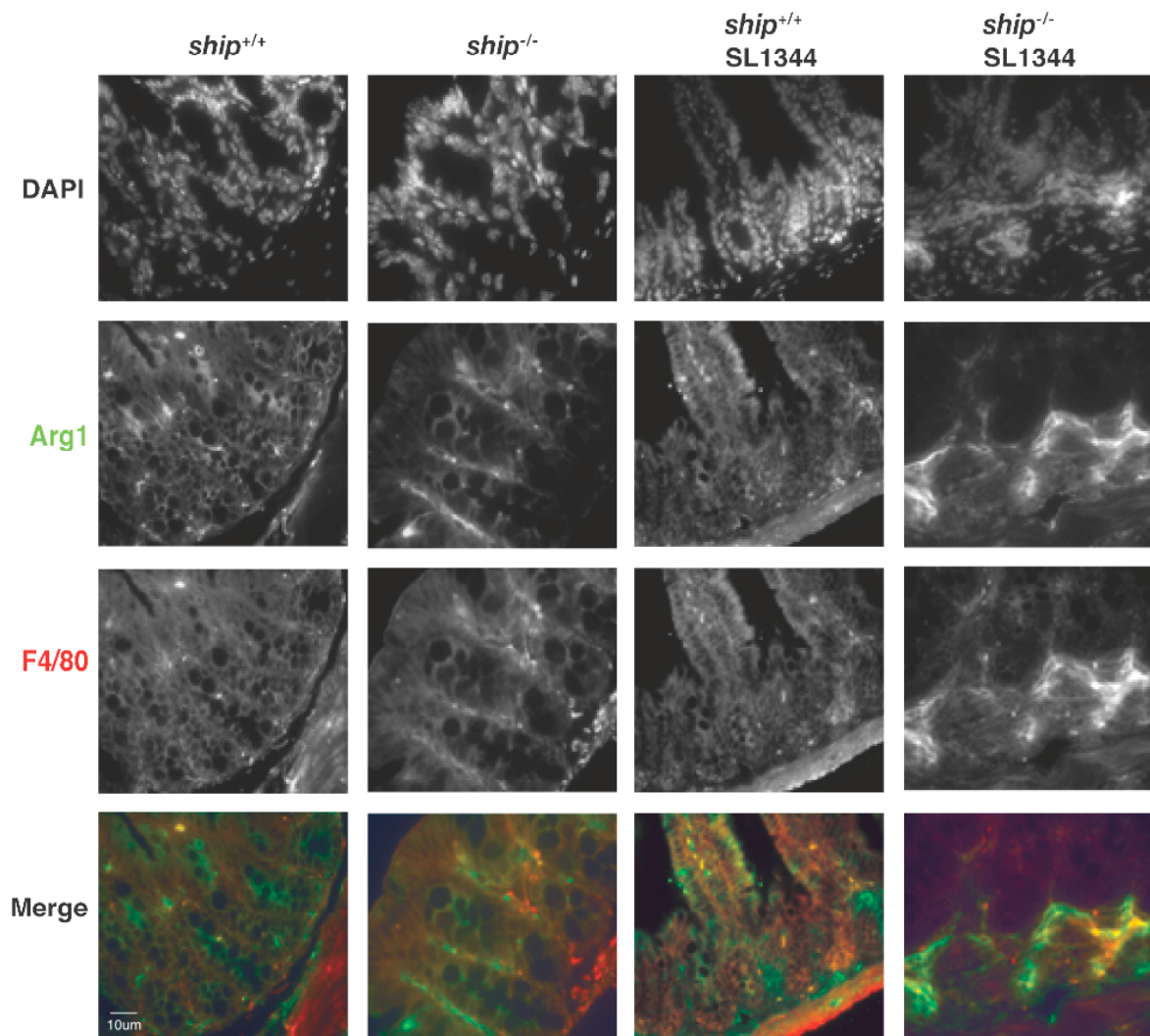
cells. YM1 and Arg1 are stained in green and mature macrophages were identified with F4/80 in red. M2 macrophages were identified as these two colors merged, in yellow (bottom color panel, Figs. 12 A and B). In addition, peritoneal macrophages from IP infected *ship*^{-/-} mice showed elevated levels of Ym1 and Arg1 compared to macrophages from uninfected and infected *ship*^{+/+} mice (Fig. 12 C). Taken together, these results suggest that macrophages in the gut and peritoneal cavity in *ship*^{-/-} mice are heavily skewed to an M2 phenotype; thus these sites may be less protected by effector cells during *Salmonella* infection.

Figure 12. M2 macrophage markers are found in the gut and peritoneal cavity of *ship*^{-/-} mice. (A and B) Sections of small intestine were taken from uninfected (*ship*^{+/+} and *ship*^{-/-}) and orally infected *ship*^{+/+} and *ship*^{-/-} mice (*ship*^{+/+} SL1344 and *ship*^{-/-} SL1344) at day 2 post-infection and stained with DAPI, the M2 macrophage markers YM1 or Arg1 (green) or and F4/80 (red) as a macrophage control. All photographs were taken under 40x magnification. (C) Peritoneal macrophages were obtained from uninfected (-SL1344) or IP infected (+SL1344) *ship*^{+/+} and *ship*^{-/-} mice at 2 days post-infection. Cells were washed, counted and lysed directly into Western Sample Buffer for protein analysis. Both bands are specific for Arg 1 (Rauh *et al.*, 2005). For A-C representative experiments are shown.

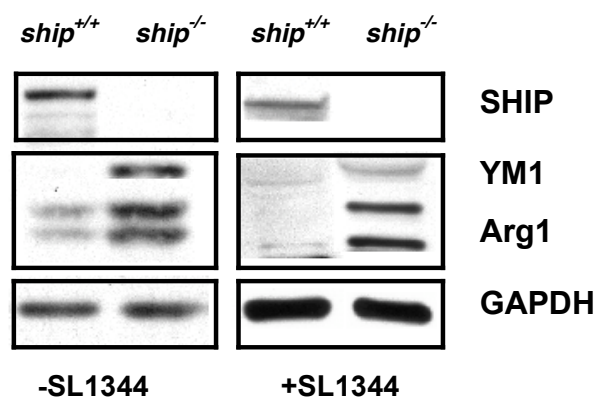
A.



B.



C.



3.2.5 Leukocyte distribution in *ship*^{-/-} mice

Because many other cell types besides macrophages are key in clearing *Salmonella* infections, and there are known differences in various immune cell populations between uninfected *ship*^{+/+} and *ship*^{-/-} mice (Brauweiler *et al.*, 2000b; Helgason *et al.*, 1998; Sly *et al.*, 2007), it was questioned whether *Salmonella* infection affected the distribution of T cells, B cells, DCs, macrophages or neutrophils found in the spleens and MLN of *ship*^{+/+} vs. *ship*^{-/-} mice. In comparing cell populations between uninfected *ship*^{+/+} and *ship*^{-/-} mice, results showed that there were significantly fewer B cells (B220+) in both the MLN (P<0.01) and spleens (P<0.001) of *ship*^{-/-} than *ship*^{+/+} mice (Tables 6 and 7, Fig. 13 B). In contrast, uninfected *ship*^{-/-} mice had significantly more macrophages (Mac-1/F4/80+) in the MLN (P<0.001) than *ship*^{+/+} mice and these levels were also elevated in the spleen, albeit not significantly (Tables 6 and 7, Fig. 13 C). These results support previously published data that showed decreased levels of B cells and increased macrophage populations in *ship*^{-/-} mice (Brauweiler *et al.*, 2000a; Helgason, 1998). Upon infection, levels of T cells (CD3+, P<0.05) and B cells (P<0.05) were significantly elevated in the spleens of *ship*^{+/+} mice, while in *ship*^{-/-} mice levels of macrophages in the MLN increased significantly (P<0.001) and dendritic cells (CD11c+) decreased significantly (P<0.05) (Tables 6 and 7, Fig. 13 A-D.) Despite these differences however, the distribution of none of these cells in the spleens and MLN of *ship*^{+/+} vs. *ship*^{-/-} mice were significantly changed upon infection with *Salmonella* when normalized against uninfected lymphocyte levels (Fig. 13 F). Thus, while inherent differences in lymphocyte populations may contribute to susceptibility to *Salmonella* infection, these phenotypes are not exaggerated in infected mice.

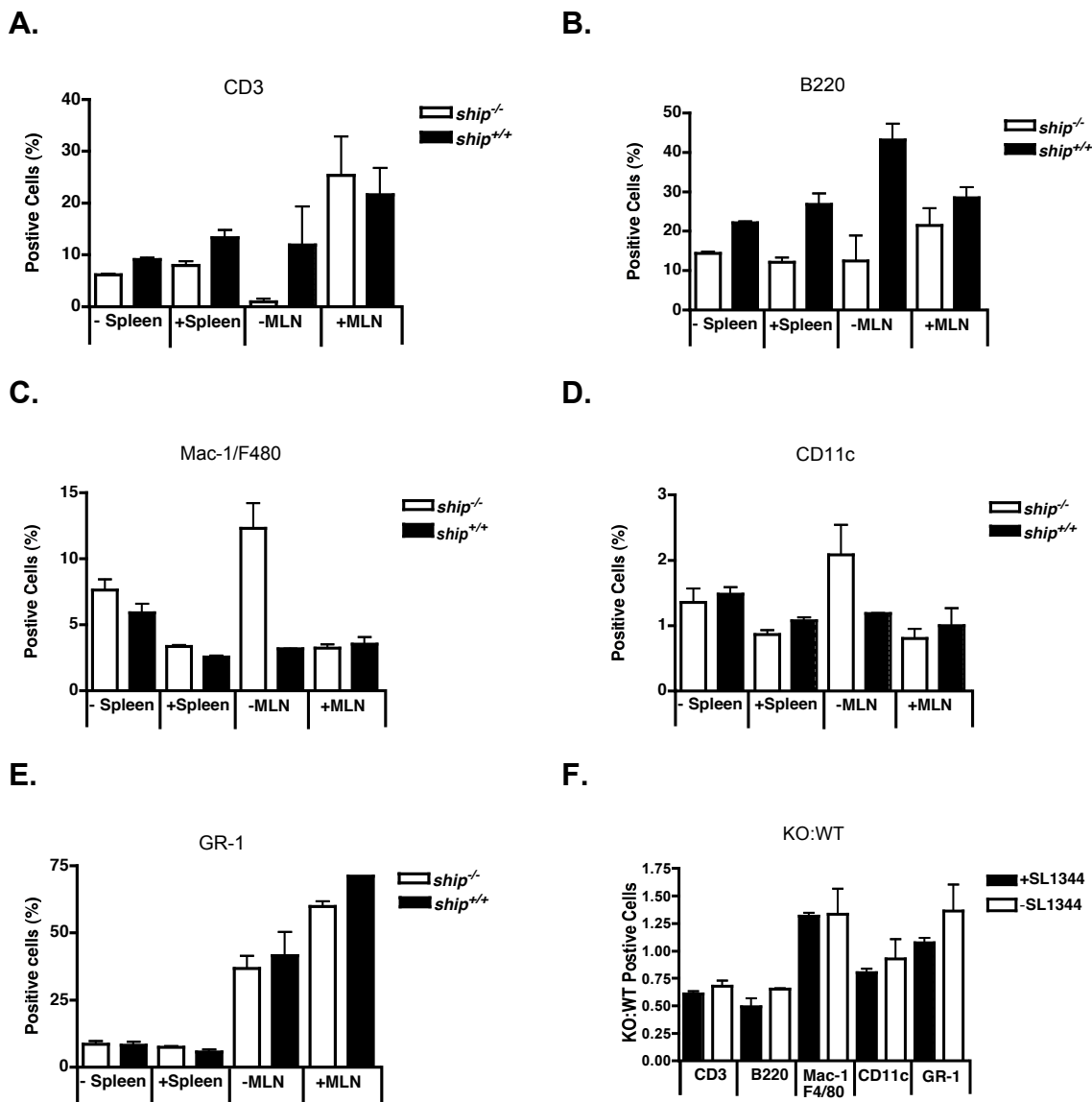
Table 6. Statistical changes in lymphocyte populations in the spleens of uninfected and *Salmonella* infected *ship*^{+/+} and *ship*^{-/-} mice. Statistical analyses were performed using a one-way ANOVA with Bonferroni post-test.

		CD3	B220	Mac-1 F4/80	GR-1	CD11c
+SL1344	<i>ship</i> ^{-/-} <i>ship</i> ^{+/+}	P<0.001 -/- < +/+	P<0.001 -/- < +/+	P>0.05	P>0.05	P>0.05
+SL1344 -SL1344	<i>ship</i> ^{-/-} <i>ship</i> ^{-/-}	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05
+SL1344 -SL1344	<i>ship</i> ^{+/+} <i>ship</i> ^{+/+}	P<0.05 - < +	P<0.05 - < +	P>0.05	P>0.05	P>0.05
-SL1344	<i>ship</i> ^{-/-} <i>ship</i> ^{+/+}	P>0.05	P<0.001	P>0.05	P>0.05	P>0.05

Table 7. Statistical changes in lymphocyte populations in the MLN of uninfected and *Salmonella* infected *ship*^{-/-} and *ship*^{+/+} mice. Statistical analyses were performed using a one-way ANOVA with Bonferroni post-test.

		CD3	B220	Mac-1 F4/80	GR-1	CD11c
+SL1344	<i>ship</i> ^{-/-} <i>ship</i> ^{+/+}	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05
+SL1344 -SL1344	<i>ship</i> ^{-/-} <i>ship</i> ^{-/-}	P>0.05	P>0.085	P<0.001 - > +	P>0.05	P<0.05 - > +
+SL1344 -SL1344	<i>ship</i> ^{+/+} <i>ship</i> ^{+/+}	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05
-SL1344	<i>ship</i> ^{-/-} <i>ship</i> ^{+/+}	P>0.05	P<0.01 -/- < +/+	P<0.001 -/- > +/+	P>0.05	P>0.05

Figure 13. Lymphocyte distribution in *Salmonella* infected *ship*^{+/+} and *ship*^{-/-} mice. Mice were left uninfected (-) or were infected orally with 1x10⁶ *S. Typhimurium* SL1344 (+) and spleens and MLN were harvested at 2 days post-infection. Cell suspensions were prepared from organs and stained for flow cytometry for T cells (A), B cells (B), macrophages (C), DCs (D), and neutrophils (E) followed by acquisition and analysis using a BD Facsclibur and FlowJo software. Graphs represent percent positivity of each cell type, and the final graph (F) was generated by creating a ratio of percent positive ko:wt cells normalized against uninfected mice. For all graphs, 2 independent experiments were performed with N=3 for both *ship*^{+/+} and *ship*^{-/-} mice and organs were pooled for cell staining. 10,000 total cells were counted for each organ and staining.



3.3 Discussion

Salmonella species pose a global threat to human health. Research using the *S. Typhimurium* mouse model of systemic salmonellosis has provided many insights into the behavior of this pathogen and the nature of immune responses required to clear intracellular infection. However, despite this progress, the ultimate cause of mortality in mice remains unknown.

One possibility is that negative regulation of immune responses during bacterial infections ultimately decides the fate of the host. For example, the ability to both sense and respond appropriately to LPS is required for resistance to *S. Typhimurium* infections (Freudenberg *et al.*, 2001; Lehner *et al.*, 2001; Xu and Hsu, 1992). In addition, regulation of pro-inflammatory pathways by enzymes such as PI3K has also been shown to play a critical role in determining the outcome of infections (Fukao and Koyasu, 2003). For example, *PI3K*^{-/-} mice show increased susceptibility to nematode infection and Gram-negative induced septic peritonitis (Fukao *et al.*, 2002b; Hirsch *et al.*, 2000) but higher resistance to *Toxoplasma* and *Leishmania* due to PI3K dependent skewing of a Th1 immune response (Fukao *et al.*, 2002a).

SHIP modulates immune homeostasis, endotoxin tolerance, PI3K signaling and macrophage inflammatory responses *in vitro*, but its role in the immune response to *in vivo* infection was undefined prior to this study. Because SHIP suppresses PI3K and PI3K has been shown to affect outcomes of various pathogenic infections, it is probable that SHIP is an important mediator in this regulatory cascade. Furthermore, since *ship*^{-/-} mice do not display endotoxin tolerance (Rauh *et al.*, 2004; Sly *et al.*, 2004) and *ship*^{-/-} macrophages can not respond to *Francisella novicida* infection (Parsa *et al.*, 2006), it was hypothesized that SHIP would be required to control *Salmonella* infection. These data show that SHIP dependent regulation of innate immune responses is critical for the control of intracellular bacterial infections *in vivo*. Furthermore, these results suggest that an excess of M2 macrophages in *ship*^{-/-} mice may be one contributing factor to exacerbated *Salmonella* pathogenesis.

Th1 mediated immunity is essential for final clearance of *Salmonella* infection both *in vivo* and *in vitro* (Eckmann and Kagnoff, 2001). For example, neutralization of IFN γ and IL-12 increases murine susceptibility to *Salmonella* infection, whereas exogenous addition of these cytokines increases host survival, and patients able to clear gastroenteritic *Salmonella* infection have higher serum levels of these cytokines (Bao *et al.*, 2000; Nauciel and Espinasse-Maes, 1992; Stoycheva and Murdjeva, 2005). Consistent with this, slightly lower levels of IL-12p70 and significantly lower levels of IFN γ were produced in *ship*^{-/-} mice during oral *Salmonella* infection (Fig. 9 A), and a similar trend was observed during IP infections as well, albeit not significant (Fig. 10 A). Importantly, this phenotype was associated with a significant increase in susceptibility to disease (Fig. 5).

In addition, levels of other innate immune cytokines, such as IL-6 and IL-10, were altered in infected *ship*^{-/-} mice. However, the role for these in *Salmonella* infection is less clear. The fact that IL-6 is upregulated during *Salmonella* infections *in vivo* and regulates PMN killing of *Salmonella in vitro* (Eckmann and Kagnoff, 2001; Nadeau *et al.*, 2002), suggests it plays a protective role against disease. Interestingly, we found IL-6 was lower in *ship*^{-/-} mice orally infected with *Salmonella* and higher in IP infected mice, suggesting that IL-6 modulation in *ship*^{-/-} mice likely plays a critical role in controlling *Salmonella* in *ship*^{-/-} mice independently of the other Th1 polarizing cytokines examined and may be heavily dependent on the route of infection. This is supported by experiments that plotted IL-6 production over the duration of oral vs. IP infections where we found that *ship*^{-/-} mice produce lower levels of IL-6 at early time points during both oral and IP infections, but not later. Therefore, SHIP may play an important role in IL-6 regulation in both oral and IP infections and at distinct times during *Salmonella* pathogenesis.

In contrast to IL-6, it has been suggested that IL-10 may be anti-protective against *Salmonella* infection (Pie *et al.*, 1996), due to its classical role as an anti-inflammatory cytokine that suppresses the functions of macrophages, DCs, NK cells and T cells (Moore *et al.*, 2001). However, more recently it has been shown that adequate IL-10 production is an essential component of the immune

response against intracellular pathogens such as *Leishmania* and *Toxoplasma* (O'Garra and Vieira, 2007; Trinchieri, 2007). Thus, the lower levels of IL-10 produced by *Salmonella* infected *ship*^{-/-} mice may also exacerbate disease.

The recruitment and activation of phagocytes within *Salmonella* infected tissues, is heavily dependent on M1, or classic macrophages; thus these cells are essential in the fight against this intracellular pathogen (Gordon and Taylor, 2005; Mantovani *et al.*, 2007). In contrast, M2 macrophages are incapable of controlling other intracellular pathogens like *M. tuberculosis* and *Leishmania*, both of which require strong Th1 immunity for clearance (Holscher *et al.*, 2006; Kahnert *et al.*, 2006). Interestingly, in the case of the *ship*^{-/-} mouse, there is a skewing of macrophages in the peritoneal cavity and lungs to an M2 phenotype and it is hypothesized that this is due to uncontrolled PI3K signaling in the absence of SHIP, or other src kinases such as Lyn and Hck (Rauh *et al.*, 2005; Xiao *et al.*, 2008a). Based on data showing dysregulated Th1 polarizing cytokines in infected *ship*^{-/-} mice, we suspected that M2 skewing was contributing to increased susceptibility to *Salmonella* infection *in vivo*. Consistent with this, peritoneal macrophages from IP infected *ship*^{-/-} animals showed a strong M2 phenotype (Fig. 12 C). During this type of infection, macrophages within the peritoneal cavity are the first innate immune cells to encounter *Salmonella* and are responsible for front line defense to prevent further spread to the blood and systemic sites (Mastroeni, 2002). During oral *Salmonella* infection however, resident tissue macrophages as well as DCs present in the Peyer's patches of the small intestine are the cells that first interact with bacteria colonizing gut tissues (McSorley *et al.*, 2002). Positive staining for Ym1 and Arg1 in these areas (Figs. 12 A and B) further supported that M2 macrophages are indeed poised at critical sites during both oral and IP *Salmonella* infection in *ship*^{-/-} mice.

Taken together, the results presented in this chapter show that the high susceptibility of *ship*^{-/-} to *Salmonella* infection seems to rely heavily on their inability to regulate cytokine responses critical to preventing infection. Furthermore, they suggest that the presence of M2 macrophages at sites of *Salmonella* infection in both IP and oral models, could be contributing to

increased susceptibility, as they are poor producers of antimicrobial molecules as well as Th1 polarizing cytokines, such as IL-12. Other contributing factors that could predispose the *ship*^{-/-} mouse to *Salmonella* infection may also be lowered B cell populations, however the relative contribution of these cells during the early time points of infection examined in both our IP and oral model suggests that poor innate defenses are most likely key to governing susceptibility to *Salmonella*.

CHAPTER 4: The role of SHIP in *S. Typhimurium* infection of macrophages
in vitro

4.1 Introduction

Macrophages are integral in the pathogenesis of *Salmonella*. While it is known now that *Salmonella* can survive in multiple cell types, originally unique to its virulence strategies were the multiple mechanisms of surviving within the macrophage and utilizing this cell type as a vehicle to spread throughout the body and cause systemic disease. As such, *in vitro* modeling of *Salmonella* pathogenesis has made extensive use of macrophages in order to determine both aspects of bacterial survival and replication as well as macrophage-dependent immune responses (Linehan and Holden, 2003).

In addition, the macrophage has also been an integral tool in studying the role of SHIP in innate immune responses. While SHIP plays an important role in cells of both the innate and adaptive immune system, its function is of particular interest in macrophages and their relationship with pathogens. This is because SHIP regulates the macrophage pro-inflammatory response to PAMPs, such as LPS and CpG DNA, as well as receptor mediated phagocytosis and phagosome formation (Sly *et al.*, 2007). Importantly, SHIP also can regulate macrophage phenotypes, which are increasingly recognized as important in determining the outcome of tumorigenesis, tissue regeneration and infectious diseases. In particular, the response of M2 macrophages is poor against most pathogens, except for protozoans, and in following they are associated with Th2 immunity. In addition, while M2 macrophages are important in wound healing and repair, they have also been implicated in the development of fibrosis during chronic inflammation as well as the development of tumors (Mantovani *et al.*, 2007). As such, they are important contributors to the development of infectious as well as autoimmune diseases and therefore may play an important role in the outcome of *Salmonella* infections.

Because SHIP deficiency has such widespread effects on both the innate and adaptive immune compartments, results using *ship*^{-/-} mice for infectious disease studies are complicated at best. Despite this, much is known regarding the importance of the macrophage in *Salmonella* infection and the role of SHIP in macrophage responses. Therefore, it follows that studying the macrophage-

dependent immune responses against *Salmonella* in a *ship*^{-/-} background is a logical place to begin assessing the relative importance of SHIP in innate responses to *Salmonella*. The results in this chapter provide insight into how SHIP deficiency alters classical macrophage responses to *Salmonella*, such as ability to limit intracellular replication, cell death and cytokine production. Importantly, these results highlight the fact that only M2 BMDMs produce lower levels of inflammatory cytokines when infected with *Salmonella*, which is what is seen *in vivo*, suggesting that these cells do indeed play an important role in the cytokine environment in the mouse during infection. Furthermore, the fact that SHIP deficiency paired with macrophage phenotype had the most dramatic effect on inflammatory cytokine production, and little effect on macrophage ability to control intracellular replication or the susceptibility of macrophages to *Salmonella* induced death, supports how critical the cytokine milieu is to preventing infection.

4.2 Results

4.2.1 Intracellular *Salmonella* replication in macrophages is independent of SHIP expression or macrophage phenotype

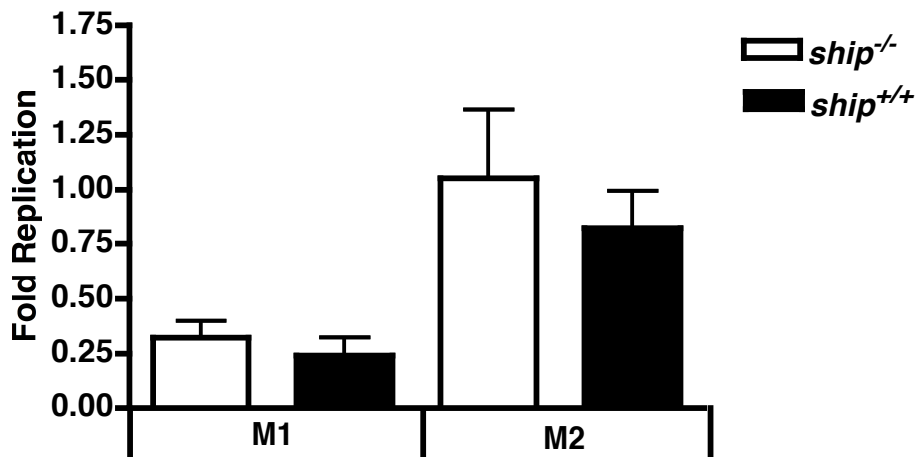
Classically activated macrophages are the primary reservoirs for *Salmonella in vivo* and modulate bacterial clearance via the production of cytokines such as IL-12p70 and IFN γ (Linehan and Holden, 2003). To investigate the role of the macrophage in the *ship*^{-/-} response to *Salmonella* infection, macrophages were derived from bone marrow of *ship*^{+/+} and *ship*^{-/-} mice under M1 or M2 derivation conditions and their responses to *Salmonella* infection were compared. To assess the capability of M1 vs. M2 macrophages to prevent intracellular *Salmonella* replication, numbers of viable *Salmonella* were assessed using a standard 24 hour gentamicin protection assay. Replication was slightly higher in BMDMs from *ship*^{+/+} and *ship*^{-/-} mice derived in the presence of mouse serum, which skews to an M2 phenotype, however these differences were not significant (Fig. 14). Importantly, there was no significant difference in intracellular *Salmonella* replication between M1 derived *ship*^{+/+} and *ship*^{-/-}

BMDMs or between M2 derived *ship*^{+/+} and *ship*^{-/-} BMDMs (Fig. 14 A), indicating that SHIP expression does not have a direct effect on internal bacterial numbers. Because *Salmonella* generally does not replicate to high numbers in BMDM however, the importance of SHIP in the macrophage bacteriostatic capability was examined by using Raw264.7 macrophages (Mastroeni and Maskell, 2006). These cells typically permit greater fold increases of bacteria over a 24 hour period and can be skewed to an M2 phenotype using IL-4 (Mantovani *et al.*, 2007). However, even in Raw cells, still no significant difference was found in *Salmonella* replication in M1 vs. M2 cells (Fig. 14 B).

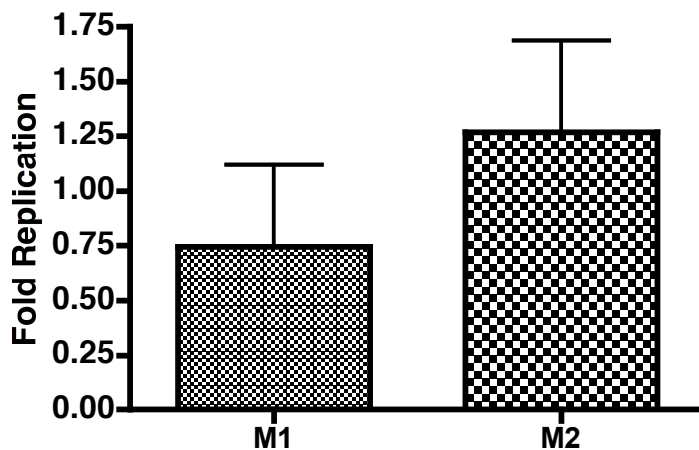
Figure 14. Changes of *S. Typhimurium* numbers in M1 vs. M2 macrophages

(A) BMDMs were obtained from *ship*^{+/+} and *ship*^{-/-} mice and derived in the presence of FBS alone (M1) or FBS + 2% mouse serum (M2) and infected with *S. Typhimurium* SL1344. (B) Raw 264.7 macrophages were grown in either DMEM + 10% FBS (M1) or skewed to an M2 phenotype by the addition of 100ng/ml IL-4 (M2) 24 hours prior to infection. For A and B cells were seeded and infected with an MOI of 10 *S. Typhimurium* SL1344 in a gentamicin protection assay with bacteria enumerated at 2 and 24 hours post-infection. Graphs represent fold replication of intracellular *S. Typhimurium* by dividing CFUs obtained at 24 hours by those at 2 hours. For both A and B three independent experiments were performed with each treatment being performed in triplicate for a total N=9 for each treatment.

A.



B.

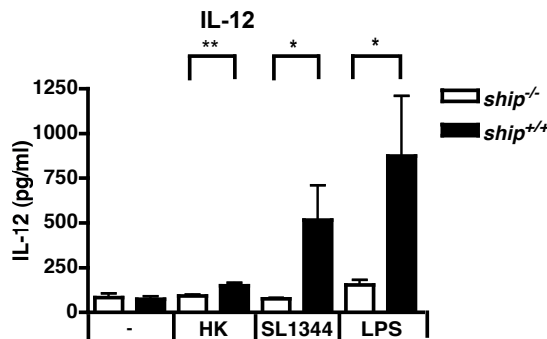


4.2.2 M2 skewing of BMDMs provides a model for macrophage function in oral *Salmonella* infection in *ship*^{-/-} mice

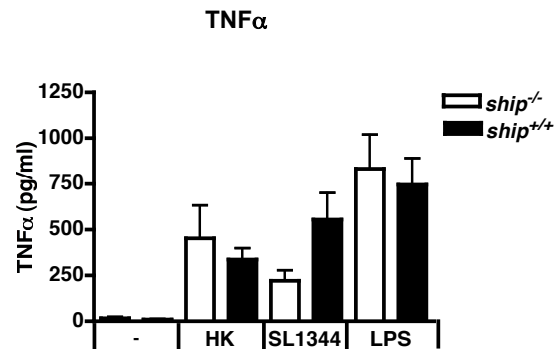
Differences in cytokine profiles produced by M1 or M2 BMDMs during infection with *S. Typhimurium* SL1344 were more apparent and dependent upon SHIP genotype. As in orally infected *ship*^{-/-} mice, *Salmonella* infected M2 derived *ship*^{-/-} BMDMs produced significantly lower levels of IL-12p70 (P=0.0201), IL-6 (P=0.017) and IL-10 (P=0.0027) than *ship*^{+/+} cells (Fig. 15). Significant differences were not found in TNF α production from *Salmonella* infected M2 BMDM *ship*^{+/+} and *ship*^{-/-} cells, or IL-6 and IL-10 production from *ship*^{+/+} and *ship*^{-/-} cells stimulated with LPS or heat-killed bacteria (Figs. 15, B, C and D). IL-12p70 production by *ship*^{+/+} cells was significantly greater than *ship*^{-/-} with LPS (P=0.0465) and heat-killed bacteria (P=0.0052, Fig. 15 A). Interestingly, lower cytokine production by *Salmonella* infected *ship*^{-/-} cells could only be seen using M2 derivation conditions; M1 derived macrophages showed significantly higher levels of IL-12p70 (P=0.0326), TNF α (P=0.0077), IL-6 (P=0.0017) and IL-10 (P=0.0143) when infected with *Salmonella* (Fig. 16). Under M1 conditions, *ship*^{-/-} and *ship*^{+/+} macrophages stimulated with LPS or heat-killed *Salmonella* showed no significant differences in production of the four cytokines tested at 8 or 24 hours post-infection (Fig. 16). Taken together, these results suggest that M2 BMDMs are better able to mimic the cytokine production seen *in vivo* during *Salmonella* infection.

Figure 15. *Salmonella* infected BMDMs from *ship*^{-/-} mice derived under M2 inducing conditions show decreased levels of inflammatory cytokines compared to *ship*^{+/+} cells. (A-D) BMDMs were obtained from *ship*^{+/+} and *ship*^{-/-} mice and were polarized to an M2 phenotype by derivation for 10 days in the presence of FBS + 2% mouse serum. Cells were seeded and either left untreated (-), infected with *S. Typhimurium* SL1344 (SL1344) or heat-killed *S. Typhimurium* SL1344 (HK) at an MOI of 10, or treated with 100ng/ml *S. Typhimurium* LPS (LPS), for 8 hours and supernatants were collected. Cytokine analysis was performed using ELISAs. For A-D three independent experiments were performed with each treatment being performed in triplicate for a total N=9 for each treatment.

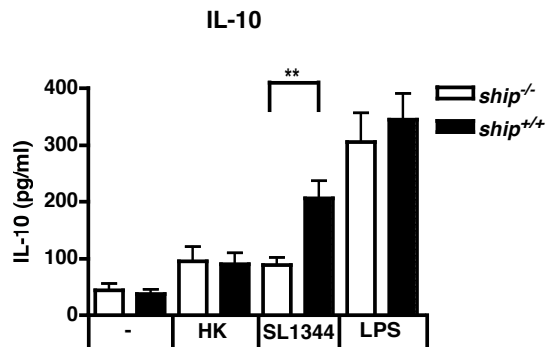
A.



B.



C.



D.

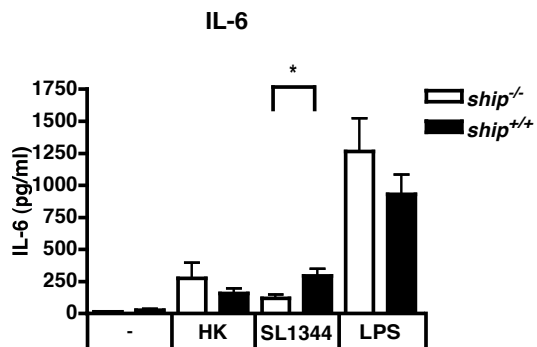
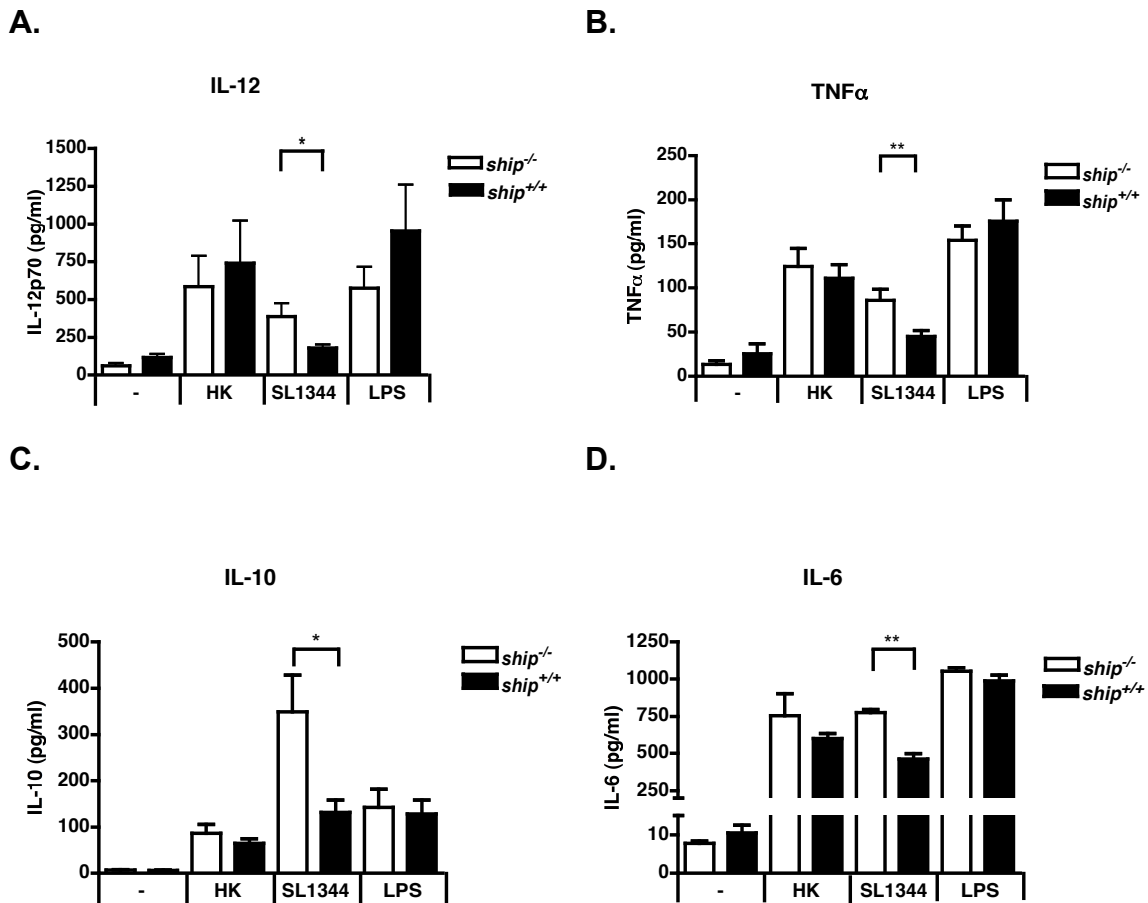


Figure 16. *Salmonella* infected BMDMs from *ship*^{-/-} mice derived under M1 inducing conditions show increased levels of inflammatory cytokines compared to *ship*^{+/+} cells. (A-D) BMDMs were obtained from *ship*^{+/+} and *ship*^{-/-} mice and were polarized to an M1 phenotype by derivation for 10 days in the presence of FBS. Cells were seeded and either left untreated (-), infected with *S. Typhimurium* SL1344 (SL1344) or heat-killed *S. Typhimurium* SL1344 (HK) at an MOI of 10, or treated with 100ng/ml *S. Typhimurium* LPS (LPS), for 8 hours and supernatants were collected. Cytokine analysis was performed using ELISAs. For A-D three independent experiments were performed with each treatment being performed in triplicate for a total N=9 for each treatment.

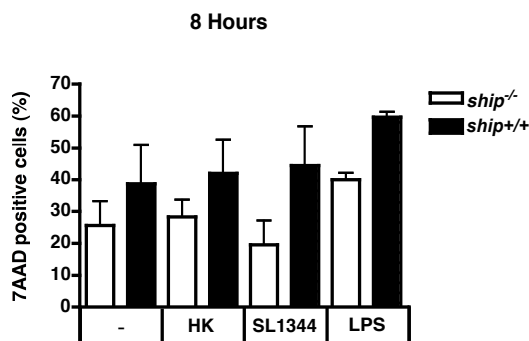


4.2.3 SHIP deficiency does not influence cell death *in vitro* during *Salmonella* infection

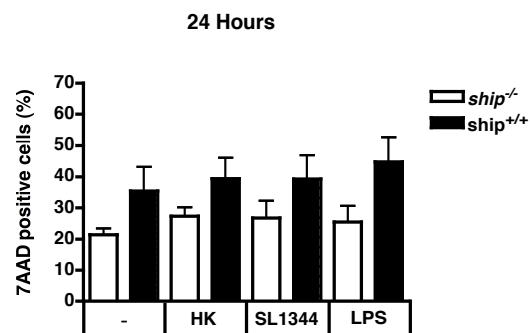
SHIP controls cell survival pathways and as well as macrophage phenotype, which can have direct effects on the longevity of a cell (Mantovani *et al.*, 2007). Thus, whether the lower cytokine production by *ship*^{-/-} BMDM was due to increased cell death in these cells was examined. As figure 17 shows, cell death was not significantly greater in *ship*^{-/-} than *ship*^{+/+} M2 BMDM at either 8 or 24 hours post-infection, suggesting that this is not the reason for lowered cytokine production upon *Salmonella* infection by *ship*^{-/-} M2 BMDM.

Figure 17. *ship*^{-/-} BMDM are not more susceptible to death upon infection with *Salmonella*. BMDMs were obtained from *ship*^{+/+} and *ship*^{-/-} mice and were polarized to an M2 phenotype by derivation for 10 days in the presence of FBS + 2% mouse serum. Cells were seeded and either left untreated (-), infected with *S. Typhimurium* SL1344 (SL1344) or heat-killed *S. Typhimurium* SL1344 (HK) at an MOI of 10, or treated with 100ng/ml *S. Typhimurium* LPS (LPS), for 8 hours (A) or 24 hours (B). Cells were collected and stained with the cell death marker 7AAD and analyzed via flow cytometry. 3 independent experiments were performed with each treatment being performed in triplicate for a total N=9 for each treatment.

A.



B.



4.3 Discussion

The high susceptibility of *ship*^{-/-} mice to *Salmonella* infection is interesting from the standpoint of both *Salmonella* pathogenesis and SHIP dependent regulation of immune responses. However, the variety of cellular responses that SHIP negatively regulates makes it difficult to assess the importance of this molecule in specific cell types during their responses to *Salmonella in vivo*. Because macrophages play such a large role in determining the outcome of *Salmonella* pathogenesis and because SHIP regulates so many macrophage responses, extrapolating data from *in vitro* *Salmonella* infections of BMDM can give insight into how these cells may contribute to infection in mice. Importantly, because SHIP skews macrophages to an M2 phenotype and these cells were found to be poised at sites of *Salmonella* infection, the relative importance of both SHIP genotype in macrophages and cell phenotype in response *Salmonella* was assessed.

M2 macrophages do not produce high levels of bactericidal mediators like reactive nitrogen or oxygen intermediates that play a role in controlling intracellular replication of *Salmonella* (Mantovani *et al.*, 2007). As expected, cells derived under M2 inducing conditions using mouse serum or IL-4 allowed slightly higher *Salmonella* replication in a 24 hour period (Fig. 14), however this difference was independent of SHIP genotype in BMDMs. In addition, consistent with other unpublished work from our laboratory, *Salmonella* did not replicate well in BMDMs from either *ship*^{+/+} or *ship*^{-/-} mice (Fig. 14 A). Interestingly, the cytokine profile produced by *ship*^{-/-} mice infected with *Salmonella* was not mimicked by *ship*^{-/-} macrophages derived under traditional M1 polarizing conditions *in vitro*. However, M2 polarized *ship*^{-/-} BMDMs infected with *Salmonella* produced a cytokine profile that most closely paralleled the one seen in orally infected *ship*^{-/-} mice. For example, M2 *ship*^{-/-} BMDMs produced significantly lower levels of IL-12p70, IL-10, and IL-6 upon *Salmonella* infection. Lower, but not significant, production of TNF α was also a hallmark sign of an M2 phenotype (Fig. 15), however this did not match what was observed *in vivo*. Importantly, reduction in cytokines was not attributed to increased cell death in *ship*^{-/-} BMDM (Fig. 17). In

contrast, experiments using conventional derivation conditions that are known to induce an M1 phenotype (Rauh *et al.*, 2005) showed that *Salmonella* infected *ship*^{-/-} M1 BMDMs produced a cytokine profile that was opposite to the one seen in *in vivo* oral *Salmonella* infections, with significantly higher levels of IL-12p20, IL-10 and IL-6 being produced (Fig. 16).

Taken together, these results showed that while M2 derived BMDMs from *ship*^{-/-} mice may not be less effective in preventing intracellular *Salmonella* replication than *ship*^{+/+} cells, they do produce very different cytokine profiles that closely mimic those seen in infected *ship*^{-/-} mice. In correlation with results showing excess M2 macrophages in the gut and peritoneal cavity during oral *Salmonella* infections, the *in vitro* behavior of M2 macrophages may provide important insight into how these cells contribute to *Salmonella* infection in *ship*^{-/-} mice.

CHAPTER 5: The role of SHIP in the intestinal inflammatory response to enteric infection

5.1 Introduction

Studying both intestinal inflammation and negative regulation of immune responses goes hand in hand. This is because the immune response in the gut must be down-regulated in the presence of commensal microbes that have the potential to stimulate strong immune responses. This regulation is a complex process that relies on balancing low pro-inflammatory mediator production with maintenance of regulatory secretory antibodies, CAMPs, and mucous products as epithelial and immune cells respond to commensal bacteria and their PAMPs. If this balance is lost and especially if there is dysregulation of pro-inflammatory and Th1, Th2 or Th17 cytokine responses, inflammatory bowel diseases (IBD), like ulcerative colitis (UC) and Crohn's disease (CD) can result (Kelsall, 2008). The severe mucosal damage inflicted by unchecked inflammation in the intestine during IBD requires wound healing in the form of collagen deposition and epithelial restitution. However, this process in CD patients is also dysregulated and fibrosis, characterized by intestinal wall thickening and uncontrolled ECM deposition, occurs (Rieder *et al.*, 2007). Fibrosis one of the most serious complication of CD, as it leads to the formation of intestinal strictures, which must be removed surgically. Unfortunately, because fibrosis is a reactive process to the chronic inflammation seen in CD, stricture formation is recurrent in most patients, even after surgery (Burke *et al.*, 2007).

SHIP has the potential to play a significant role in the development of inflammation and fibrosis in the intestine; however this has not been previously examined. SHIP controls the pro-inflammatory status of macrophages that is strongly implicated in the development of IBD (Kelsall, 2008; Sly *et al.*, 2007). For example, ROS production by macrophages is a requirement for the tissue destruction that characterizes IBD and macrophages of CD patients have high levels of NF- κ B transcription and downstream production of TNF α (Kelsall, 2008; Kruidenier and Verspaget, 2002). Similarly in *ship*^{-/-} macrophages, other groups have found high levels of TNF α are produced in response to LPS. In addition, TGF β , which is one of the most potent pro-fibrotic mediators in the body, regulates macrophage pro-inflammatory responses via SHIP (Sly, 2004).

Also, SHIP actively represses the generation of M2 macrophages, and these cells have been implicated in playing a role in fibrosis development (Sangaletti *et al.*, 2003). Therefore, since SHIP regulates many cellular processes required to prevent intestinal inflammation, it is probable that *ship*^{-/-} mice might develop intestinal pathology in response to commensals or enteric pathogens that mimics IBD.

Oral infection of mice with *S. Typhimurium* provides an excellent model to study human typhoid infection, which is a systemic, febrile disease. However, unlike in humans, where NTS such as *Typhimurium* cause gastroenteritis, this phenotype is not seen in murine models unless the microbiota is altered with antibiotic treatment prior to infection (Bartel, 2003). However, during the course of experiments examining susceptibility of *ship*^{-/-} mice to oral *S. Typhimurium* infection, these mice exhibited severe swelling of the small intestine primarily located in the ileum. The experiments presented in this chapter therefore, were designed to investigate the nature and cause of intestinal inflammation in the *ship*^{-/-} mouse. Histology and examination of cytokines showed that the increase in size of the ileum was in fact due to excessive inflammation. In addition, signs of fibrosis, such as collagen deposition and the presence of high levels of pro-fibrotic factors, like TGFβ and MCP-1 were also found in the ileum. Importantly, results showed that this phenotype is not specific to *Salmonella* infection, as *Citrobacter rodentium*, *Helicobacter*, as well as heat-killed bacteria and LPS also led to inflammation.

5.2 Results

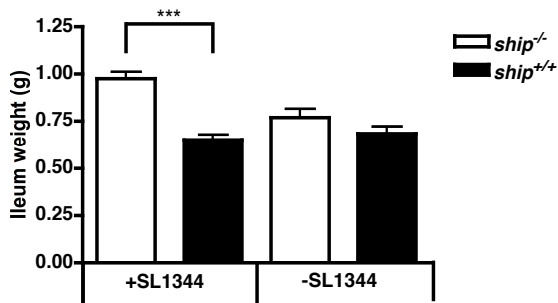
5.2.1 *Salmonella* infected *ship*^{-/-} mice have highly inflamed small intestines

The nature of *S. Typhimurium* infection in mice is a systemic disease, minimal intestinal pathology is seen (and limited to Peyer's patches) and typically the small intestine is not highly colonized by bacteria. However, upon infection of *ship*^{-/-} mice with oral *S. Typhimurium* and without pre-treatment of mice with

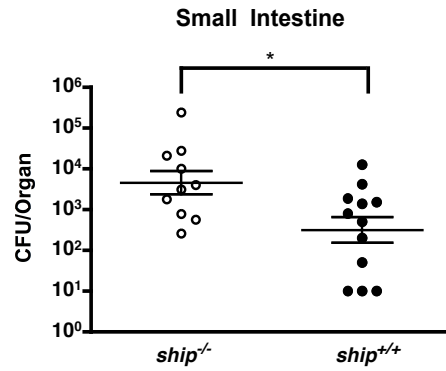
streptomycin, what appeared to be severe swelling of the ileum was observed. This phenotype was accompanied by significant increases in small intestinal weight (Fig. 18 A) as well as high colonization with *S. Typhimurium* at two days post-infection (Fig. 18 B). Upon examination of small intestinal sections by hematoxylin and eosin Y (H&E) histology, which stains nucleic acids, ribosomes and cellular proteins, it was clear that intestines from *ship*^{-/-} mice exhibited increased size and weight due to massive infiltration of inflammatory cells and edema (Fig. 18 C). A pathology scoring system that assessed inflammation in the lumen, surface epithelium, mucosa and submucosa of the ilea, showed a more quantitative difference between *ship*^{-/-} and *ship*^{+/+} ilea (Fig. 18 D). The inflammation seen in these H&E slides correlated with a significant increase in the inflammatory cytokines TNF α (P<0.0001) and MCP-1 (P=0.0087) in the small intestine taken at day two post-infection (Fig. 19). Importantly, uninfected *ship*^{-/-} mice and *ship*^{+/+} mice showed no inflammation by histology and cytokine levels were lower than the detectable limit (Fig. 18 E).

Figure 18. *Salmonella* infection in *ship*^{-/-} mice leads to inflammation of the ileum. *ship*^{+/+} and *ship*^{-/-} mice were infected orally with 1x10⁶ *S. Typhimurium* SL1344 and were sacrificed at 2 days post-infection. (A and B) Small intestines were removed from uninfected (-SL1344) and *Salmonella* infected (+SL1344) mice, weighed and homogenized for bacterial counts. (C and D) Prior to homogenization, 0.25 micron sections of ilea were excised and stained with H&E for pathology and scored. (E) H&E sections of ilea from uninfected mice. For A, B and D, 3 independent experiments were performed with a total N=12 for both *ship*^{+/+} and *ship*^{-/-} mice. For C and E, representative experiments are shown.

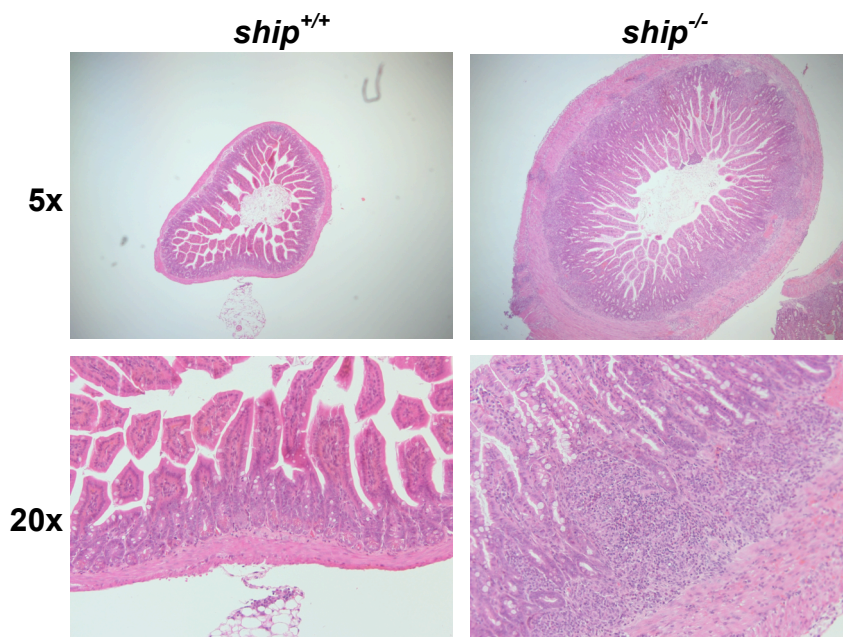
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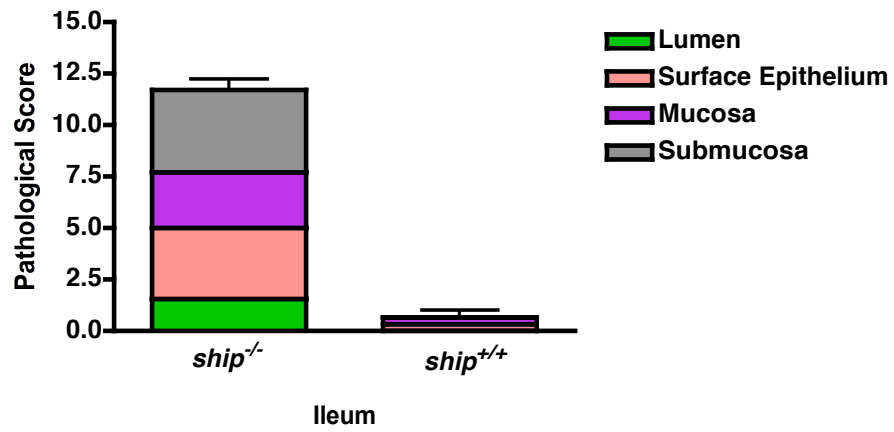
B.



C.



D.



E.

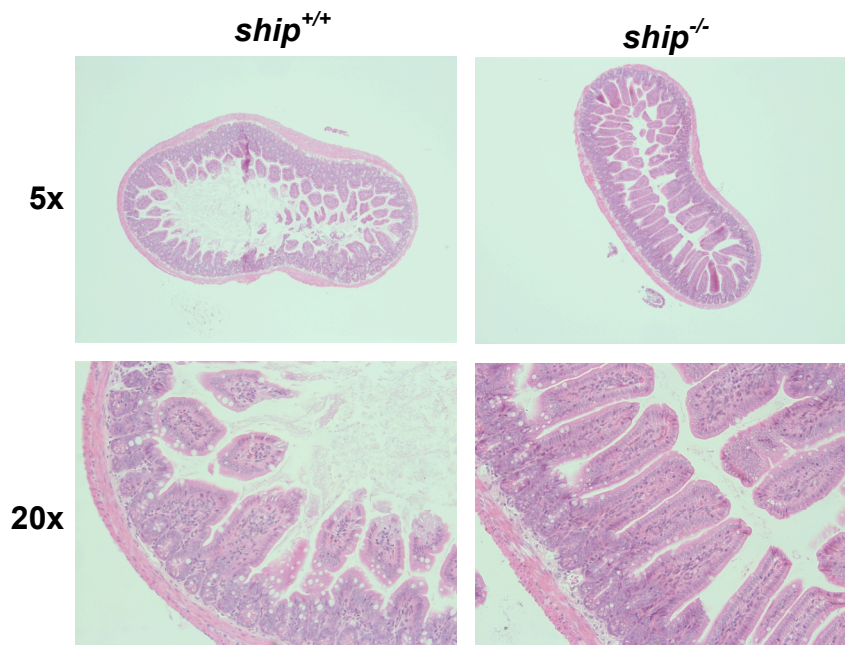
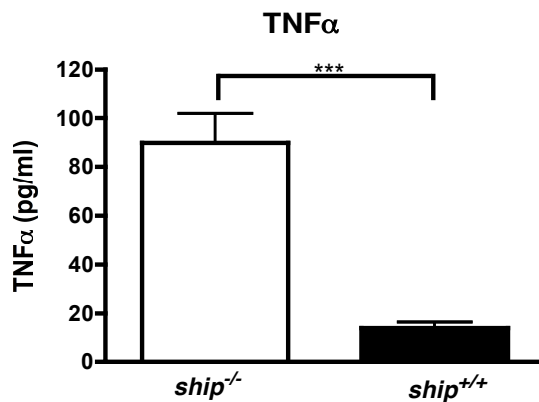
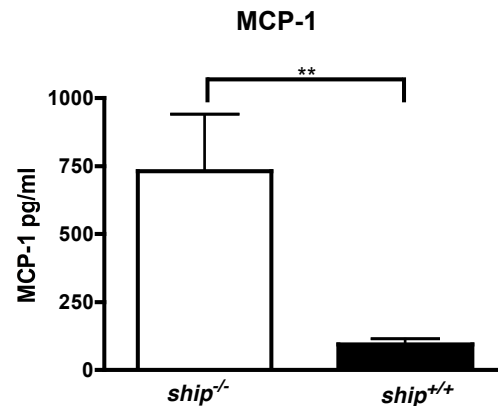


Figure 19. $\text{TNF}\alpha$ and MCP-1 are increased in $\text{ship}^{-/-}$ small intestines during *Salmonella* infection. $\text{ship}^{+/+}$ and $\text{ship}^{-/-}$ mice were infected orally with 1×10^6 *S. Typhimurium* SL1344 and were sacrificed at 2 days post-infection. (A and B) Small intestines were homogenized and supernatants were analyzed for pro-inflammatory cytokines using ELISAs. For A and B, 3 independent experiments were performed with a total N=12 for both $\text{ship}^{+/+}$ and $\text{ship}^{-/-}$ mice.

A.



B.



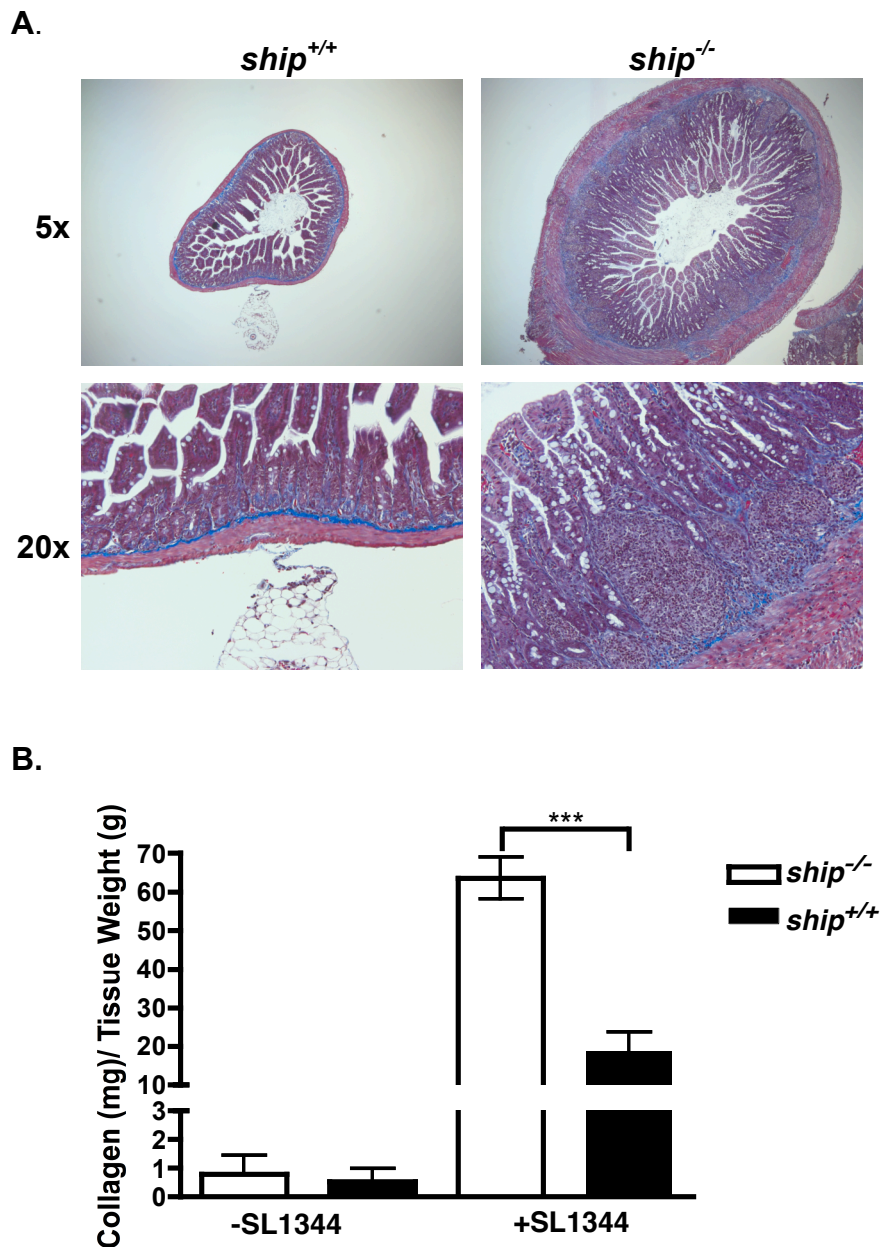
4.2.2 Fibrosis is a consequence of inflammation induced during *Salmonella* infection in the small intestine of $\text{ship}^{-/-}$ mice

Fibrosis is the formation of excess connective tissue in the small intestine and it is a severe complication of IBD. In IBD, fibrosis occurs as a direct result of chronic or recurrent inflammation of the intestine and it is a reactive process-the more inflammation occurs, the more fibrotic tissue develops (Rieder *et al.*, 2007). Because of the high degree of intestinal inflammation caused by *S. Typhimurium* infection in $\text{ship}^{-/-}$ mice and the infiltration of M2 macrophages, which are associated with fibrotic tissue generation, to this area (Chapter 3), it was questioned whether fibrosis was occurring in the ilea of *Salmonella* infected $\text{ship}^{-/-}$ mice. Masson's trichrome histology stain was used to determine collagen deposition in the ilea of $\text{ship}^{+/+}$ and $\text{ship}^{-/-}$ mice orally infected with *S. Typhimurium*. As Figure 20 A shows, collagen (blue stain) deposition in $\text{ship}^{-/-}$ mice is widespread in the submucosa as well as muscularis propria, whereas in

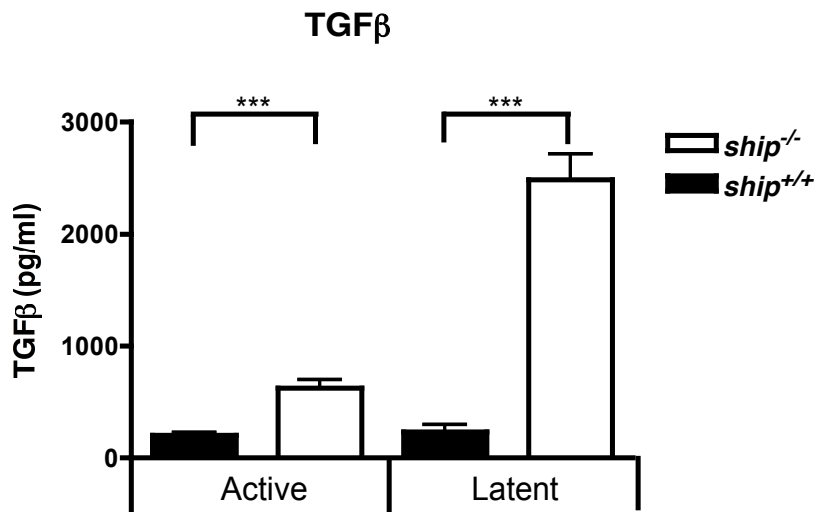
ship^{+/+} mice it remains tightly compacted to a thin layer at the muscularis mucosae. To quantify collagen deposition, sections of ilea were digested in pepsin and soluble collagens were assessed by a sircol dye based collagen assay. Results of these tests showed a significant increase in collagen levels upon infection of *ship*^{-/-} mice, whereas in *ship*^{+/+} mice levels stayed steady (P<0.001, Fig. 20 B).

Several pro-fibrotic mediators are essential for the development of fibrosis in the intestine, including TGF β and MCP-1. TGF β is the most potent pro-fibrotic indicator in the intestine, as it regulates induces the production of other pro-fibrotic mediators and cell types, like collagen transforming growth factor (CTGF), insulin-like growth factor (IGF) and Th17 cells, as well as reduces the production of matrix metalloproteases (MMP) which are necessary for collagen breakdown (Rieder *et al.*, 2007). MCP-1 is a critical chemoattractant for the inflammatory cells that are responsible for tissue destruction that precedes fibrosis, and was already found to be elevated in the intestines of *ship*^{-/-} infected mice (Fig. 19). Because TGF β is necessary for the induction of fibrosis, its presence was assessed in ilea from *Salmonella* infected *ship*^{+/+} and *ship*^{-/-} mice. Figure 20 C shows that both latent and active forms of TGF β are significantly higher (P=0.0001) in the ilea of *ship*^{-/-} than *ship*^{+/+} mice. Both uninfected *ship*^{+/+} and *ship*^{-/-} mice produced levels of TGF β below the level of detection. Taken together, these results suggest that despite the short duration of inflammation in the ileum induced by oral *Salmonella* infection in *ship*^{-/-} mice, pro-fibrotic mediators are present and collagen deposition leading to fibrosis can occur.

Figure 20. Fibrosis is a consequence of small intestinal inflammation in *ship*^{-/-} ilea during *Salmonella* infection. *ship*^{+/+} and *ship*^{-/-} mice were infected orally with 1x10⁶ *S. Typhimurium* SL1344 and were sacrificed at 2 days post-infection. (A) Masson's Trichrome stain of ilea sections taken at 2 days post-infection. (B) 0.5 cm of Ileum was removed at 2 days post-infection and pepsin soluble collagen was assessed using a standard collagen assay. Small intestines were removed and homogenized at 2 days post infection and supernatants were analyzed via ELISA for TGFβ (C). In A, a representative experiment is shown. For B and C, 3 independent experiments were performed with a total N=12 for both *ship*^{+/+} and *ship*^{-/-} mice.



C.



4.2.3 Development of inflammation and fibrosis in the ileum of *ship*^{-/-} mice is not specific to *Salmonella* infection

A large contributing factor to the development of IBD is the immune response against microflora that colonize the intestine (Duchmann *et al.*, 1995). In fact, there is a direct correlation between the number of bacteria in the colon, terminal ileum and caecum and the development of CD; these areas are where the commensal population is most dense and where CD manifests in the gastrointestinal tract (D'Haens *et al.*, 1998). For the most part, it did not seem that *ship*^{-/-} mice were susceptible to inflammation induced by commensal bacteria, since the majority of uninfected mice presented with no intestinal pathology (Fig. 18 E). However, a few uninfected mice were found with enlarged ilea, raising the possibility that the intestinal inflammatory response in *ship*^{-/-} mice was not specific to *Salmonella* infection.

To assess to what degree other pathogens or products could be affecting intestinal pathology in *ship*^{-/-} mice, contamination of mice with *Helicobacter* species as a potential cause of intestinal inflammation in "uninfected" controls was investigated. There are 8 species of *Helicobacter* that infect the mouse intestinal tract and contamination of commercial and academic colonies is

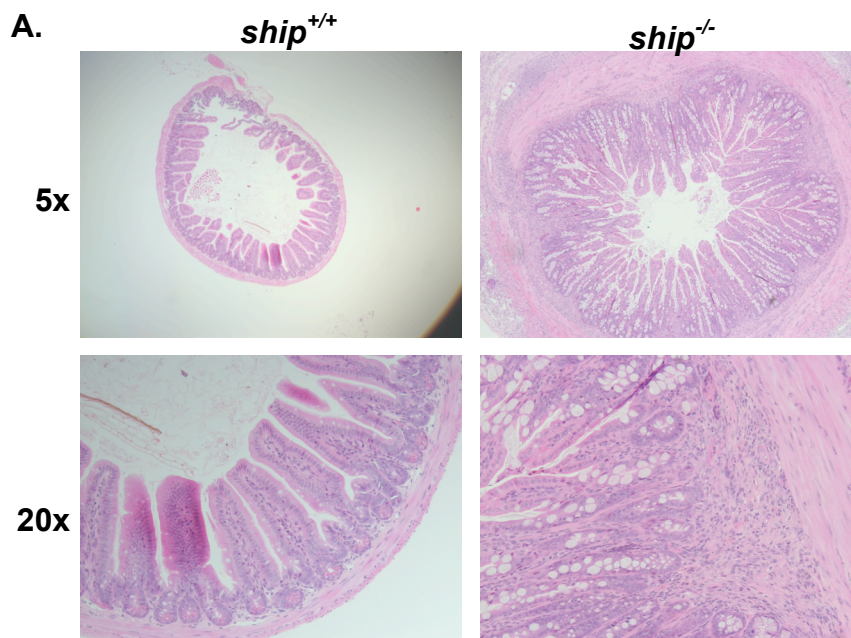
extremely high. Importantly, in many immunocompromised mouse strains, colonization with typically non-pathogenic *Helicobacter* sp. can induce inflammatory disease (Whary and Fox, 2006). PCR analysis specific for all 8 *Helicobacter* species was done by RADIL on pooled stool samples from *ship*^{-/-} and *ship*^{+/+} mice. Results showed infection with three different species of *Helicobacter*, *hepaticus*, *typhlonius* and *rodentium*. Based on correspondence with collaborators at the British Columbia Cancer Research Centre (BCCRC), the introduction of *Helicobacter* infection in rederived *ship*^{-/-} mice corresponded with the development of small intestinal inflammation, suggesting that this infection was a probable cause for inflammation seen in my "uninfected" controls.

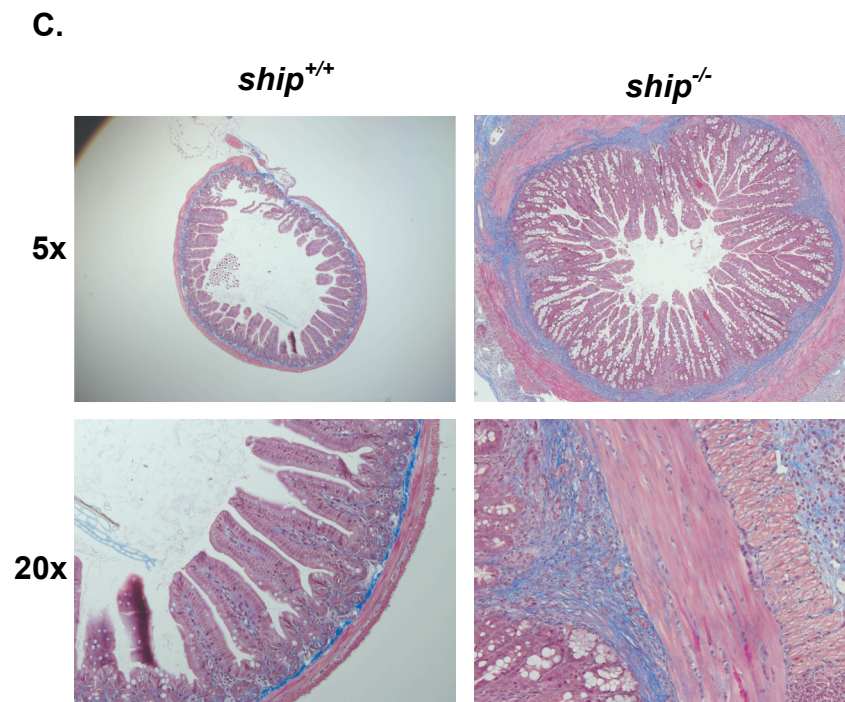
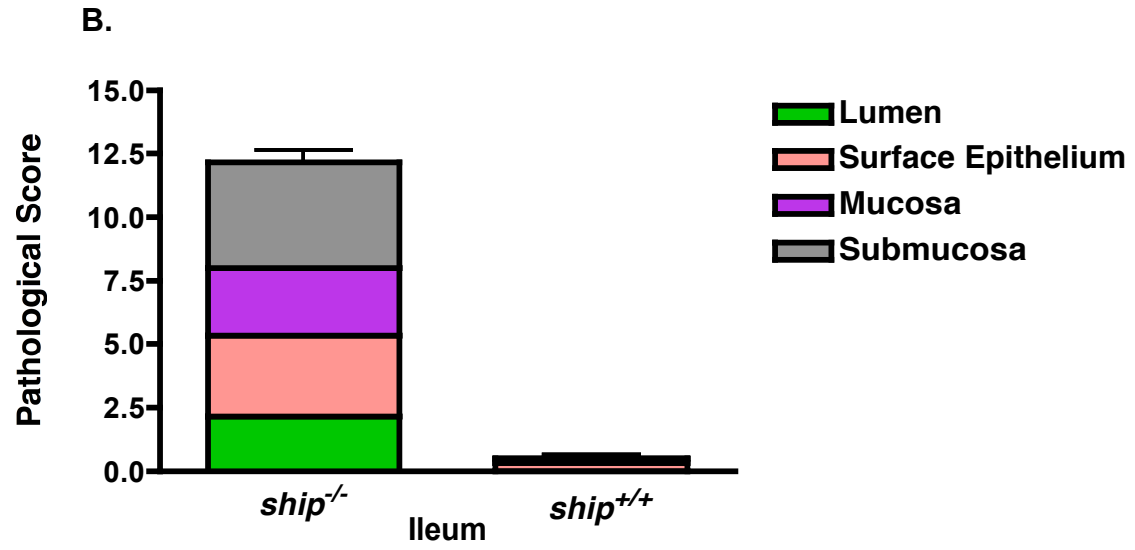
After confirmation that both *ship*^{-/-} and *ship*^{+/+} mice were infected with *Helicobacter*, it was essential to define if co-infection was a prerequisite for *Salmonella*-induced intestinal inflammation. *Helicobacter*-free *ship*^{-/-} and *ship*^{+/+} mice, that were rederived from another mouse colony and housed in a separate facility as well as screened as *Helicobacter* negative by PCR, were infected with *S. Typhimurium* orally and intestinal pathology was examined at two days post-infection. As figure 21 shows, *Helicobacter*-free *ship*^{-/-} mice also showed severe inflammation as well as collagen deposition in the ileum. Importantly, *ship*^{-/-} and *ship*^{+/+} mice free of *Helicobacter* and left uninfected did not show intestinal pathology (Fig. 21 D).

To investigate whether another enteric pathogen was capable of inducing intestinal inflammation, *ship*^{-/-} mice were infected with *Citrobacter rodentium* and intestinal pathology was monitored at seven days post-infection, which is the time it typically takes for this bacterium to colonize the colon (Mundy *et al.*, 2005). While *ship*^{-/-} mice were not more susceptible to colonization by *C. rodentium* (Fig. 8) and did not become sick upon infection, they did display severe inflammation and fibrosis of the ilea after 7 days (Fig. 22). Taken together, these results show that both intracellular and extracellular enteric pathogens, as well as commensal species like *Helicobacter* are not tolerated by the immune response in the small intestine of *ship*^{-/-} mice.

Interestingly, infection with viable bacteria was not a requirement for the induction of intestinal inflammation in *ship*^{-/-} mice, since infection with both heat-killed *S. Typhimurium* as well as *C. rodentium*, resulted in similar intestinal pathology as live infections (Fig. 23). In addition, gavage with LPS from *S. Typhimurium* was also able to induce inflammation after two days, suggesting that strong PAMPs on the microbe surface are sufficient to initiate the inflammatory response in these mice (Fig. 23).

Figure 21. *Helicobacter* infection is not a prerequisite of *Salmonella* induced intestinal inflammation. *Helicobacter*-free *ship*^{+/+} and *ship*^{-/-} mice were infected orally with 1×10^6 *S. Typhimurium* SL1344 and were sacrificed at 2 days post-infection. 0.25 cm of ilea were excised and pathology was scored based on H&E histology staining (A and B). (C) Masson's trichrome stain for collagen deposition in infected mice. (D and E) H&E and Masson's trichrome histology on ilea from uninfected *Helicobacter*-free mice. For A, C, D and E representative experiments are shown. For B, 3 independent experiments were performed with a total N=12 for both *ship*^{+/+} and *ship*^{-/-} mice.





D.

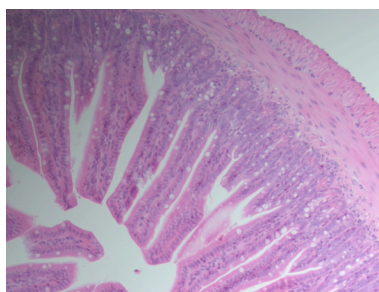
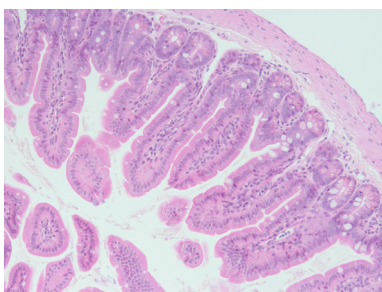
ship^{+/+}

ship^{-/-}

5x



20x



E.

ship^{+/+}

ship^{-/-}

5x



20x

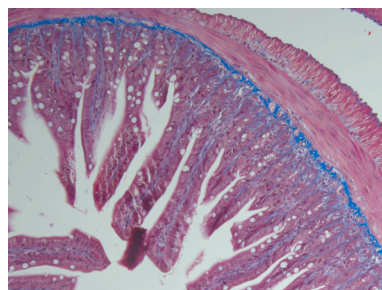
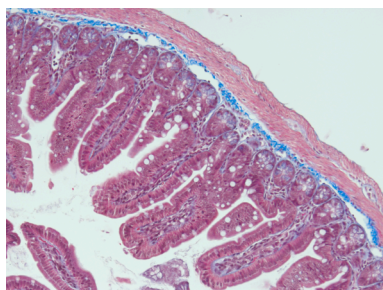
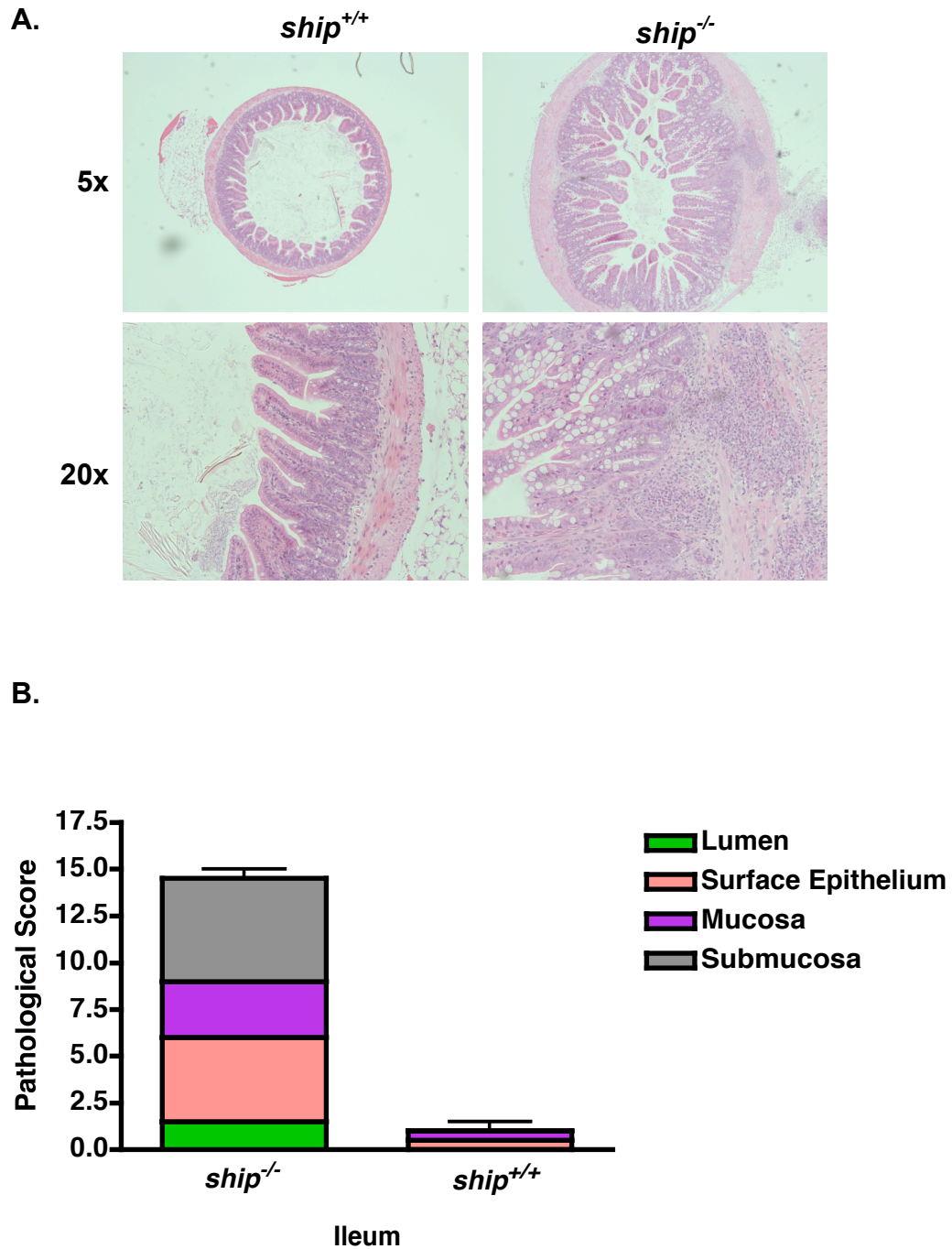


Figure 22. *Citrobacter rodentium* induces intestinal inflammation in *ship*^{-/-} mice. *ship*^{+/+} and *ship*^{-/-} mice were infected orally with 1x10⁸ *C. rodentium* DBS100 and were sacrificed at 7 days post-infection. 0.25 cm of ileum were excised and pathology was scored based on H&E histology staining (A and B). (C) Masson's trichrome stain for collagen deposition. For A and C, representative experiments are shown. For B, 3 independent experiments were performed with a total N=12 for both *ship*^{+/+} and *ship*^{-/-} mice.



C.

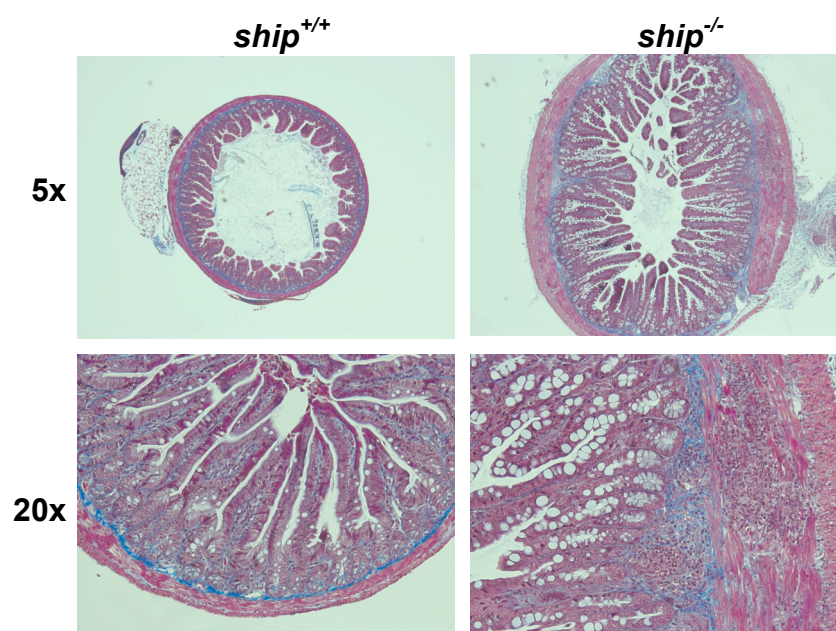
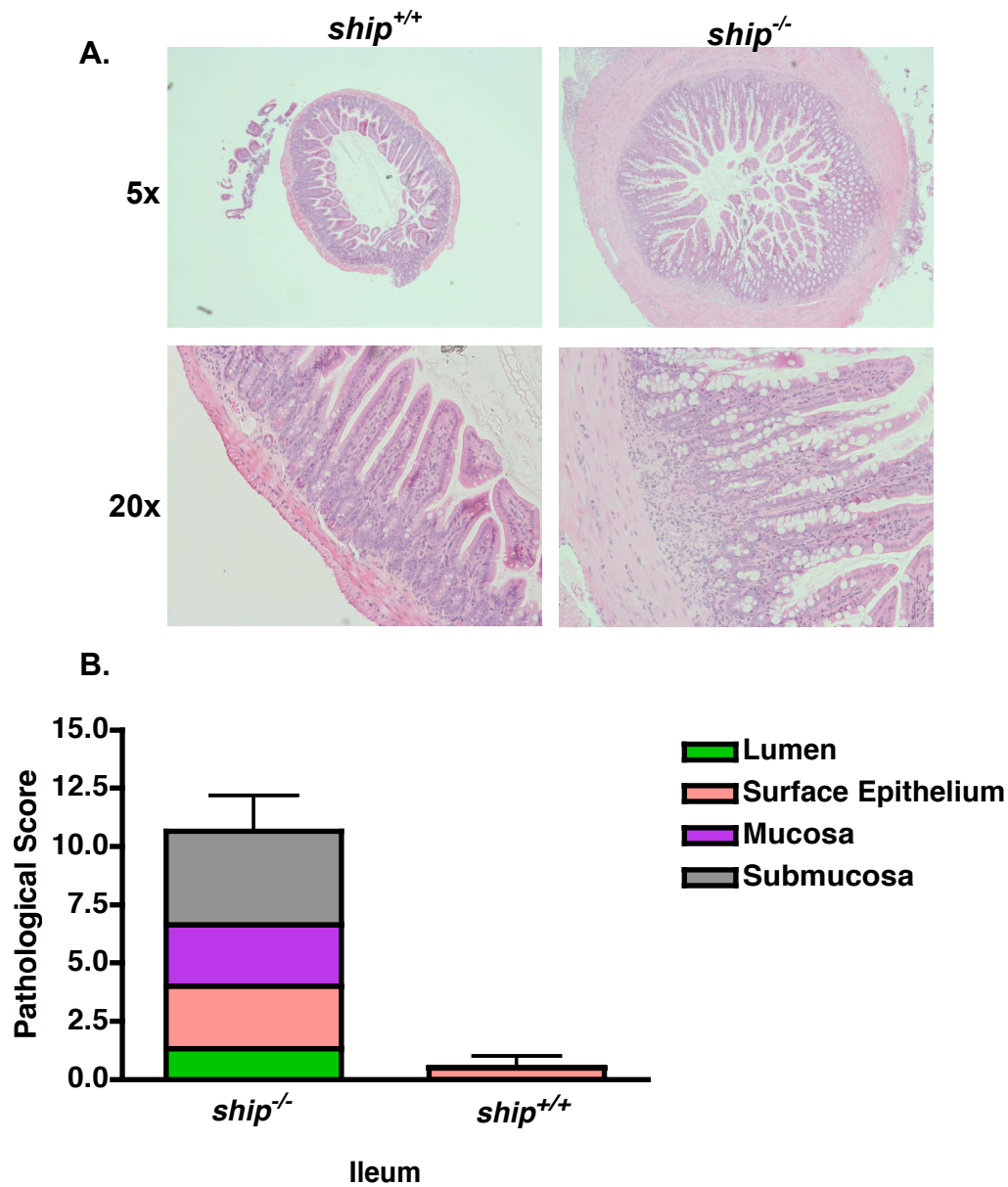
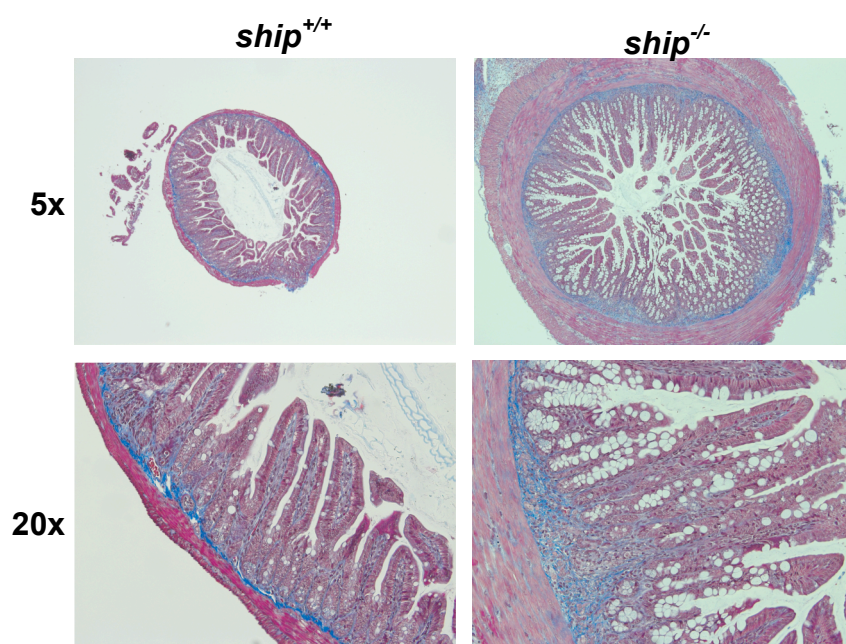


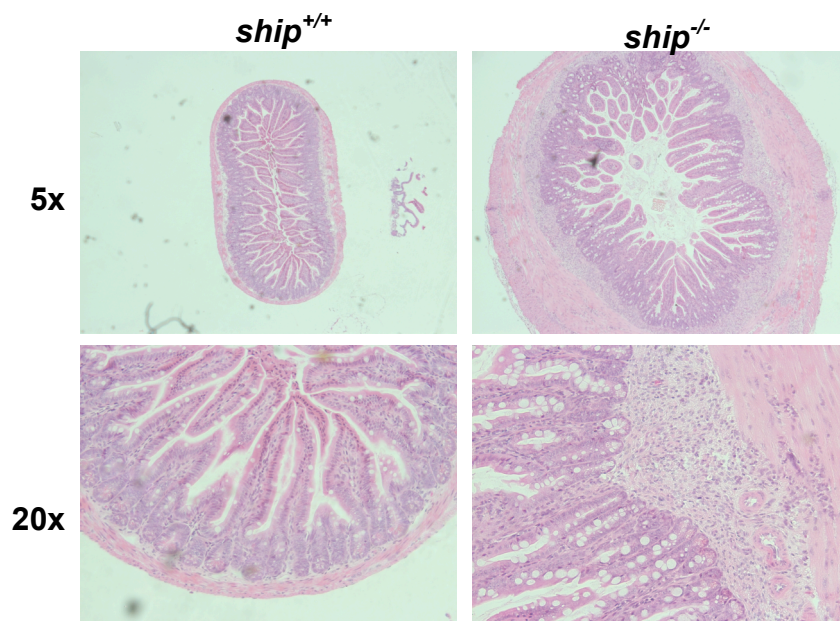
Figure 23. Heat-killed bacteria and LPS can induce intestinal inflammation in *ship*^{-/-} mice. *ship*^{+/+} and *ship*^{-/-} mice were infected orally with 1x10⁶ heat-killed *S. Typhimurium* SL1344 or 1x10⁸ heat-killed *C. rodentium* DBS100 and were sacrificed at 2 or 7 days post-infection, respectively. 0.25 cm of ileum were excised from *ship*^{-/-} mice infected with heat-killed *Salmonella* (A-C) or *Citrobacter* (D-F), stained with H&E, pathology scored and Masson's trichrome stained, respectively. (G-H) *ship*^{+/+} and *ship*^{-/-} mice were treated orally with 50mg/kg *S. Typhimurium* LPS, sacrificed at 2 days post infection and ilea sections were stained for H&E and Masson's trichrome histology, respectively. For A, C, D, F, G and H representative experiments are shown. For B and E, 3 independent experiments were performed with a total N=12 for both *ship*^{+/+} and *ship*^{-/-} mice.

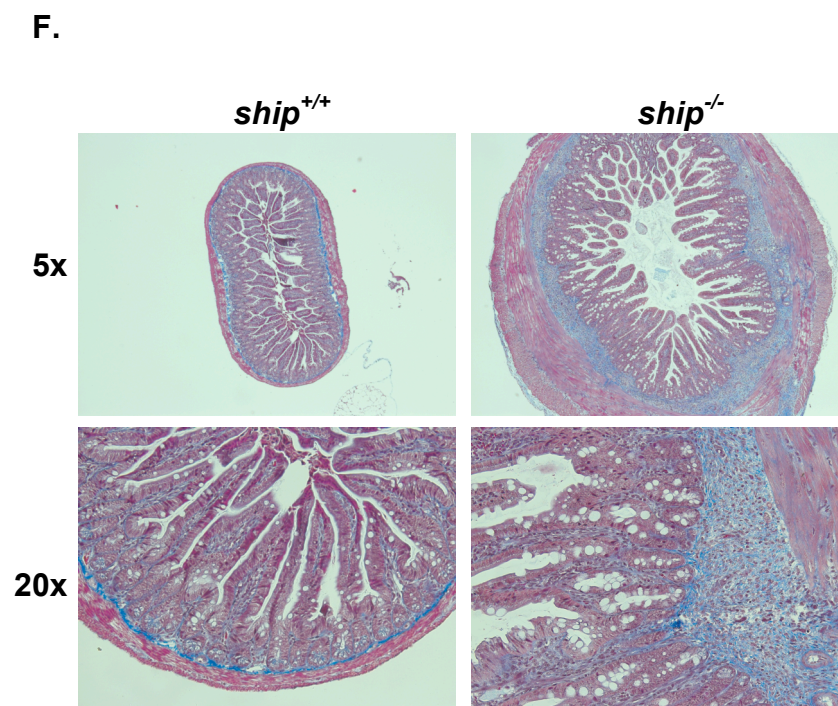
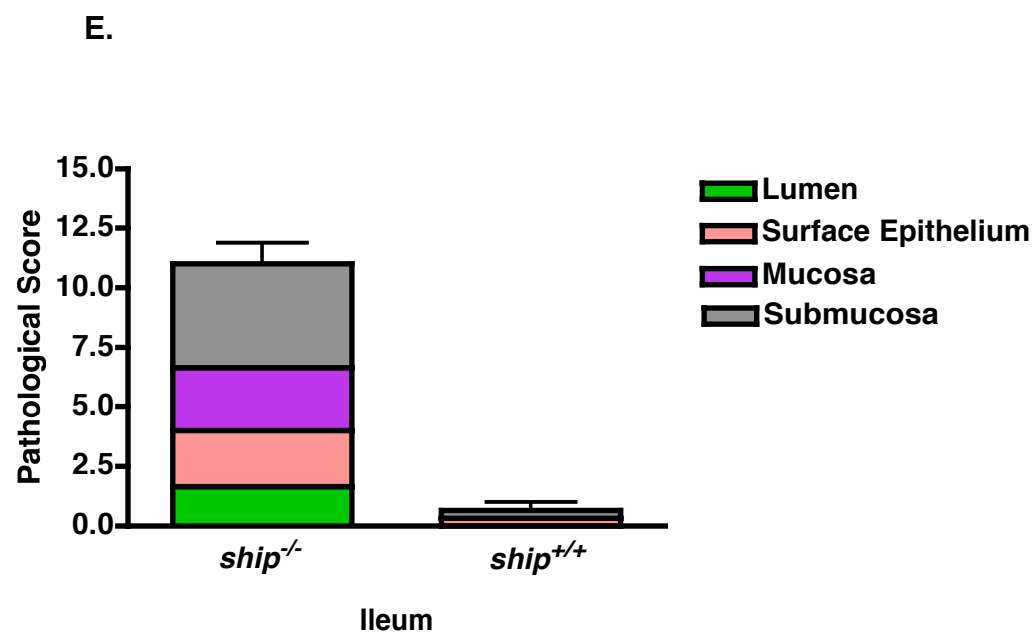


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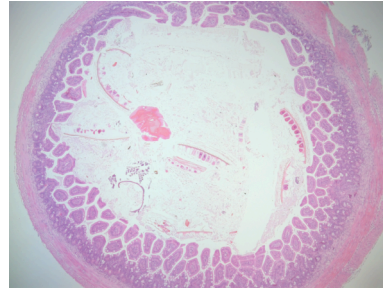


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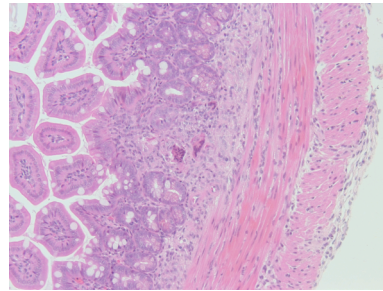
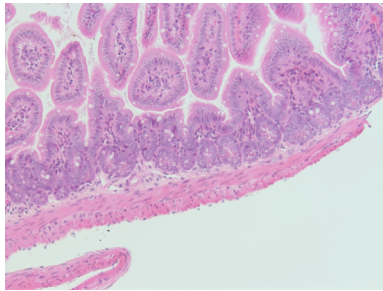
ship^{+/+}

ship^{-/-}

5x



20x

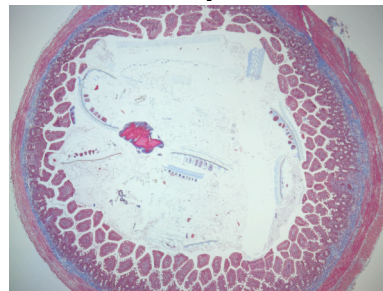


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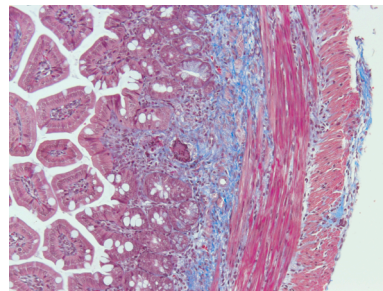
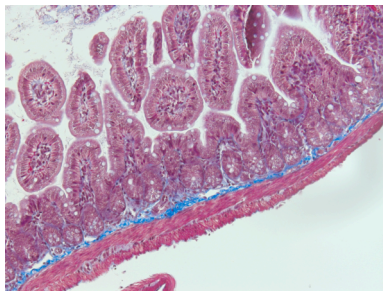
ship^{+/+}

ship^{-/-}

5x



20x



5.3 Discussion

IBD results from a dysregulation of both inflammatory and wound healing processes in the intestines. While pathology seen in UC is typically restricted to the intestinal mucosa, CD is a transmural disease where inflammation is found throughout the mucosa, submucosa and muscle layers. Fibrosis, characterized by increased collagen deposition and disruption or expansion of the muscle layer of the intestine, is the most severe complication of CD. During fibrosis, overexpansion of fibroblasts and mesenchymal cells together with overexpression of adhesion molecules and pro-fibrotic factors, leads to the formation of excess ECM, thickening the gut wall and decreasing elasticity (Rieder *et al.*, 2007). In fact, despite the available treatments for IBD, such as immunomodulatory therapies, antibiotics and probiotics, fibrotic stricture formation in CD patients still leads to significant mortality (Van Assche *et al.*, 2004).

Susceptibility to IBD is controlled by a number of factors, including the gut microflora, environmental stimuli, genetics and immunity (Bouma and Strober, 2003). Immune responses generated against normal flora in the intestine are highlighted as one of the major causes for IBD development (Elson *et al.*, 2005). In a healthy individual, inflammation induced by commensal bacteria is minimized by the epithelium and immune cells residing in the lamina propria. In addition to the production of CAMPs and bacterial permeability inducing protein (BPI) that directly attack bacterial membranes (Eckmann, 2005), epithelial cells produce mucus, which provides a physical barrier against bacteria and their products from moving to deeper tissues (Podolsky, 1999). Secretory IgA is also an important defense at the epithelial surface against commensal organisms (Macpherson *et al.*, 2000). If the epithelial barrier is breached however, PMNs and macrophages in the lamina propria are responsible for clearing bacteria by traditional innate responses such as phagocytosis and lysosomal degradation (Kelsall, 2008). The differentiation of T regulatory cells in this region is also of critical importance in regulating immune responses (Bouma and Strober, 2003). However, in IBD patients, both the epithelial barrier and immunity in the lamina propria is

compromised. Overproduction of pro-inflammatory mediators by innate immune cells, such as TNF α and high mobility group box protein 1 (HMGB-1), as well as mast cell proteases, break down epithelial tight junctions and can cause epithelial cell apoptosis that leads to increased intestinal permeability (Bruewer *et al.*, 2003; Jacob *et al.*, 2005; Liu *et al.*, 2006). Uncontrolled positive feedback of IL-23, Th1 cytokines, IL-17 and TGF β produced by innate immune cells in the lamina propria are then responsible for the uncontrolled inflammation and fibrosis development seen in CD (Fantini *et al.*, 2006; Uhlig *et al.*, 2006; Wilson *et al.*, 2007).

Few effective animal models are available that mimic human IBD, in part because mice are not susceptible to developing fibrosis in the intestine (Pucilowska *et al.*, 2000). This resistance to inflammatory disease correlates well with the fact that mice do not develop the same intestinal pathology upon infection with enteric bacteria, such as *S. Typhimurium*, that is seen in humans. However, inflammation and fibrosis can occur upon infection with *Salmonella* after oral pre-treatment with streptomycin. Chronic infection in this model results in both severe inflammation and fibrosis development in the caecum and colon that mimics pathology and cytokine responses that are seen in CD patients (Grassl *et al.*, 2008). In addition, these data show that inflammation also occurred in *ship*^{-/-} mice upon infection with *Salmonella*, however antibiotic pretreatment was not required. This phenotype raises the interesting questions of how SHIP controls the inflammatory response in the intestine, and whether *ship*^{-/-} mice may be used as an effective model to study IBD.

SHIP deficiency may affect the development of intestinal inflammation and fibrosis by altering immune defenses of both the epithelial layer and lamina propria. For example, results showed that there is significantly more TNF α in the small intestine of *Salmonella* infected *ship*^{-/-} mice, and TNF α contributes to both the breakdown of tight junctions as well as epithelial cell apoptosis in IBD (Bruewer *et al.*, 2003). Degranulation of mast cells and the subsequent release of proteases is also important for increasing the permeability of the epithelial barrier in IBD (Jacob *et al.*, 2005). Because SHIP sets the activation threshold of mast

cells and limits degranulation, lack of this regulation in the intestine during infection has the potential to increase inflammation (Huber, 2000). Finally, because *ship*^{-/-} mice have lower levels of B cells, the production of secretory IgA, which is critical in preventing bacteria from breaching the epithelium, may be diminished (Brauweiler *et al.*, 2000a; Macpherson *et al.*, 2000)

In the lamina propria, elevated levels of both TNF α and MCP-1 are required for inflammation (Kelsall, 2008). The fact that both cytokines were significantly higher in *ship*^{-/-} mice may be a direct effect of the lack of negative regulation on LPS dependent pro-inflammatory signaling in *ship*^{-/-} macrophages. Furthermore, the combination of the presence of these cytokines with the fact that SHIP negatively regulates PMN recruitment to these mediators may further exacerbate inflammation in the lamina propria (Sly *et al.*, 2007). In *ship*^{-/-} mice, fibrosis may be occurring quickly because of the hyper-responsiveness of may be dependent on the high density of M2 macrophages in the small intestine during infections. While M2 macrophages are required for normal wound healing processes, they are also found at sites of fibrotic tissue development (Mantovani *et al.*, 2007). Thus, the combination of uncontrolled inflammatory responses and M2 skewing in the *ship*^{-/-} gut could provide an ideal environment for rapid collagen deposition.

Whether *ship*^{-/-} mice could be used to study IBD will depend on the similarities and differences between the inflammatory phenotype seen in the *ship*^{-/-} ileum and factors implicated in the development of CD in other mouse models and in humans (Table 8). The primary paradigm for the development of IBD, specifically CD, is the overproduction of Th1 polarizing cytokines, such as IL-12 and IFN γ as well as TNF α , paired with a deficient regulatory T cell response. More recent studies have also shown that these cytokines as well as IL-6 regulate the development of Th17 T cells and the production of IL-17 and IL-23, which are present in both CD patients as well as mouse models of IBD (Kelsall, 2008). Fibrosis development is characterized by increased collagen deposition that requires pro-fibrotic factors like TGF β (Rieder *et al.*, 2007). In the *ship*^{-/-} mouse however, while TGF β and MCP-1 levels were high, TNF α was the

only pro-inflammatory cytokine seen to be elevated in the intestines. *ship*^{-/-} mice had no significant increase in IL-6 in the small intestine, which acts in a positive feedback loop in the development of Th17 responses that characterize IBD. Importantly, it is unknown what levels of IL-17 or IL-23 are in the *ship*^{-/-} intestine, highlighting the fact that more work needs to be done to better characterize intestinal inflammation in this model and compare it to IBD. Nonetheless, the occurrence of inflammation in the intestine of *ship*^{-/-} mice provides new avenues to explore the importance of SHIP in gut immunity as well as alternative mechanisms that may lead to the development of IBD.

Table 8. Similarities and differences between intestinal inflammation seen in the *ship*^{-/-} mouse compared to IBD.

SIMILAR TO IBD	DIFFERENT THAN IBD	UNKNOWN
High TNF α	IL-12 not elevated	IL-17 levels
High MCP-1	IFN γ not elevated	IL-23 levels
High TGF β	IL-6 not elevated	Presence of regulatory T cells in small intestine
Increased collagen deposition	Located in ileum instead of caecum and colon	Th2 cytokine levels

CHAPTER 6: DISCUSSION

6.1 Regulation of anti-*Salmonella* immunity by SHIP

The importance of negative regulators during immune responses to pathogens has been highlighted using various mouse models. PI3K, SHP-1, LYN and PTEN^{+/-} mice all have severe impairment of immune homeostasis and are susceptible to various pathogens, such as *Mycobacterium tuberculosis*, *Leishmania*, *Pseudomonas*, as well as cancers. However, in some models, such as SHP-1^{-/-} mice infected with cytomegalovirus, deficient immune modulation can actually have a protective effect against disease (Veillette *et al.*, 2002). Indeed, *ship*^{-/-} mice have many immune characteristics that could both increase or decrease their susceptibility to pathogens. For example, myeloproliferation and decreased activation thresholds in both innate and adaptive immune cells might prime immune responses, while skewing of macrophage, NK and T cell responses towards an inhibitory phenotype would easily predispose *ship*^{-/-} mice to infection.

Data presented here confirmed that the latter of these scenarios is true; *ship*^{-/-} mice are highly susceptible to *Salmonella* infection. Contributing factors in this susceptibility are poor induction of Th1 polarizing cytokines, M2 macrophage skewing at sites of infection, and possibly hyper-responsive immunity in the gut to either the bacteria themselves, or LPS. However, what remains unclear is when SHIP dependent regulation of immune responses is most important during *Salmonella* infection. Potentially, SHIP deficiency may impact susceptibility to *Salmonella* either i) during systemic phases of disease; an idea which is supported by the fact that *ship*^{-/-} mice were not susceptible to *Citrobacter rodentium*, ii) during systemic responses to LPS or iii) in the gut, where *Salmonella* clearly induces an overzealous innate response. Regardless of where SHIP plays a more substantial role, it is clear that it, like so many other negative regulators, is an essential component in our defenses against infectious disease.

6.2 The role of SHIP in systemic Salmonellosis

The simple lack of negative regulation does not necessarily equal increased susceptibility to disease—it seems that the nature of the pathogen and cell responses governed by the regulator are both important factors in determining pathogenesis. This fact is exemplified in the *ship*^{-/-} mouse, which was found to be highly susceptible to both oral and IP *Salmonella* infection, yet was as well colonized by *Citrobacter rodentium* as were *ship*^{+/+} mice. While both pathogens cause enteric disease, the main distinguishing features between the two is that in mice, *S. Typhimurium* is an intracellular pathogen that causes systemic disease, while *Citrobacter* is not. In addition, being an attaching and effacing pathogen, *Citrobacter* targets epithelial cells of the colon to establish infection, whereas *Salmonella* rapidly breaches the epithelial barrier and disseminates to systemic organs within phagocytic cells (Mastroeni and Maskell, 2006; Mundy *et al.*, 2005).

Salmonella establishes systemic infection by secreting a variety of effector molecules via its TTSS that manipulate the host cell environment and make it conducive to bacterial growth (Gal-Mor and Finlay, 2006). Once the epithelium is breached, SPI-2 effectors are essential for creating the SCV and allowing *Salmonella* replication within macrophages and a variety of other cell types. Importantly, macrophages and DCs are the primary cell types that shuttle bacteria from the gut to systemic organs where infection foci are established, such as the liver, and spleen (Mastroeni and Maskell, 2006). In the *ship*^{-/-} mouse, it was found that there are significantly higher bacterial counts in these sites during infections. SHIP deficiency may allow for higher bacterial numbers in the liver and spleen in two ways. Firstly, *ship*^{-/-} mice suffer from severe over-proliferation of myeloid cells; it is well known that *ship*^{-/-} mice have higher levels of circulating macrophages and myeloid DC's, and this leads to the pulmonary inflammation that eventually kills the mice (Helgason *et al.*, 1998). Therefore, it is possible that higher CFU counts are found in systemic sites simply because there are more myeloid cells to infect. However, whether these cells have the same phagocytic capacity towards *Salmonella*, or if they are able to home

properly from the gut to systemic sites during infection, remains unknown. Certainly, the phenotype of the macrophage could be an important factor in determining whether more cells would equal greater levels of infection.

Secondly, the bacteriostatic capabilities of *ship*^{-/-} macrophages or other cells that permit intracellular replication might be impaired. From studies presented in chapter 4, it is clear that at least M2 macrophages do not produce cytokines necessary to initiate bacteriostatic responses against *Salmonella*, such as IL-12 and IL-6. These "adaptive/innate" responses of macrophages are essential for preventing mortality in mice and are also important for the bacteria to establish a persistent, yet non-fatal infection of systemic organs (Fig. 2). The bacteriostatic capability of macrophages *in vivo* is exemplified by the fact that *Salmonella* does not replicate to high numbers per cell in systemic sites (Sheppard et al., 2003). In the *ship*^{-/-} mouse however, it is unknown to what degree and in what cell types *Salmonella* may be replicating, or whether M2 cells are truly the culprits of the lower levels of IL-12, IL-6 and IFN γ that are produced during infection. However, the fact that M2 cells were found at sites of infection suggests that they may provide a reservoir where unchecked bacterial replication can occur. This is supported by *in vitro* data showing not significant, but elevated, bacterial replication in M2 vs. M1 macrophages. Furthermore, while it is known that M2 cells are highly phagocytic for cellular debris (Mantovani, 2007), we do not know whether these cells behave similarly towards pathogens or whether *Salmonella* can replicate to high numbers per M2 cell *in vivo*.

Adaptive immunity is also strictly governed by SHIP in both T and B cells and it is critical in the control and eventual clearance of systemic Salmonellosis. However, the question of whether T and B cells control susceptibility to *Salmonella* in the *ship*^{-/-} mouse model is interesting since most *ship*^{-/-} mice die from infection much earlier than classic adaptive responses are required. Despite this fact however, the lower number of B cells in *ship*^{-/-} mice could affect systemic Salmonellosis in that B cells, apart from producing antibodies, can act as APC's as well as harbor replicating *Salmonella* (Mastroeni et al., 2000). In particular, the

APC function of B cells during *Salmonella* infection may be essential for imparting protection from disease.

6.3 The role of SHIP in endotoxin tolerance during systemic Salmonellosis

In survival experiments, *ship*^{-/-} mice were highly susceptible to infection with live *Salmonella* via both oral and IP infection routes, but did not become ill from injection or gavage with heat-killed bacteria. These experiments were designed to assess the relative importance of LPS present in infection inoculum in causing death. However, they can not answer whether LPS present at systemic sites from replicating bacteria is the real cause of mortality seen in *Salmonella* infected *ship*^{-/-} mice. The fact that systemic levels of TNF α , a major inducer of endotoxic shock, were consistently higher in *Salmonella* infected *ship*^{-/-} mice suggests this may be the case. The highly sensitive phenotype of *ship*^{-/-} macrophages to LPS, at least *in vitro*, would easily contribute to this cytokine profile during infection and could lead to endotoxin mediated death.

Despite these factors however, there is evidence to suggest that LPS sensitivity may not be the cause of increase susceptibility to *Salmonella* in *ship*^{-/-} mice. For example, other classic mediators of toxic shock, like IL-12 and IFN γ , were significantly lower in *ship*^{-/-} mice during *Salmonella* infections. In addition, LPS from *Citrobacter rodentium* infection was not sufficient to cause death in *ship*^{-/-} mice, although this is most likely due to the fact that *Citrobacter* does not migrate to systemic sites. Most importantly however, is the fact that the phenotype of macrophages in *ship*^{-/-} mice is skewed to an M2 phenotype. The major role of these cells in the body is not to combat bacterial infections, but rather to remodel tissues after destructive immune responses. While results presented in chapter 5 showed that M2 BMDM from both *ship*^{+/+} and *ship*^{-/-} mice can respond to LPS, *ship*^{-/-} M2 cells did not produce more inflammatory cytokines than *ship*^{+/+} cells with this stimulus. This contrasts with the response of M1 *ship*^{-/-} BMDM to LPS, since it has been shown that they hypersecrete pro-inflammatory cytokines upon stimulation (Sly *et al.*, 2004). Therefore, despite the fact that there are many more circulating myeloid cells in the *ship*^{-/-} mouse, a majority of

them are of an M2 phenotype with a dampened pro-inflammatory response so might not contribute to endotoxin sensitivity. Results showing that both *ship*^{-/-} mice as well as *ship*^{-/-} M2 BMDM produce lower levels of IL-12, IFN γ and IL-6 support this idea. Further biochemical analysis of M2 macrophage responses to LPS *in vitro* would also shed light on how these cells function during bacterial infections.

In addition, it has been found that SHIP is upregulated in response to other TLR ligands, such as CpG DNA but not double stranded RNA, indicating that its function may be to regulate MyD88 dependent signaling in innate immune cells (L. Sly and G. Krystal, submitted). Therefore, in the *ship*^{-/-} mouse during *Salmonella* infection there are potentially other bacterial products besides LPS that have the capacity to overstimulate immune cells and mediate death. Again however, it is unknown how these stimuli signal in M2 macrophages and what types of immune responses they are capable of mounting in the presence of these PAMPs. Thus, while M2 cells may play a large role in allowing increased bacterial dissemination and replication in systemic sites, whether they can contribute to shock caused by LPS or other PAMPs remains unknown. Clearly, many more biochemical experiments are needed *in vitro* on *ship*^{-/-} M2 macrophages in order to tease out the relative importance of these cells during bacterial infections.

6.4 The role of SHIP in gut immunology

The importance of SHIP regulating gut immunology is highlighted by the fact that *ship*^{-/-} mice develop severe inflammation in the ileum during challenge with either enteric bacteria or LPS. This response is rapid and strong; by two days post-challenge the ilea are filled with inflammatory cells and there is already deposition of collagen in both the muscle layers and submucosa. Most likely, this is due to both a breakdown of integrity in the epithelium and poor immune responses in the lamina propria to invading bacteria or bacterial products. Indeed, SHIP is required to negatively regulate the production of both pro-inflammatory and pro-fibrotic factors that were found to be elevated in the guts of

ship^{-/-} mice, such as TNF α , MCP-1 and TGF β , and it of course regulates the M2 phenotype of macrophages, which also play a role in the development of fibrosis. However, many questions remain regarding this inflammatory phenotype. For example, why does SHIP play such an important role in regulating immunity in the small intestine but not in the caecum or colon? Why isn't there an overactive immune response to bacterial products from resident microflora in the *ship*^{-/-} gut? Finally, what impact does gut inflammation have on the development of systemic Salmonellosis?

SHIP deficiency alters immune homeostasis because it regulates the distribution of different types of immune cells in the body as well as the strength and duration of their responses to various stimuli. Therefore, how immune cells are distributed in the *ship*^{-/-} mouse may explain why the small intestine is so susceptible to inflammation yet the rest of the GI tract is not. For example, M2 macrophages were seen in the small intestine during *Salmonella* infection and maybe these cells either do not differentiate in, or home to, other sites of the gut as readily. Other cell populations, such as regulatory T cells, are extremely important in mediating tolerance in the gut to microbial stimuli (Bouma and Strober, 2003). It is unknown what the distribution of these cells is in the small intestine or colon of *ship*^{-/-} mice, although it is known that *ship*^{-/-} mice have overall higher populations of both myeloid suppressor and regulatory T cells in systemic organs (Kashiwada *et al.*, 2006). If this is the case however, while these cells may show "regulatory" phenotypes by cell surface marker or protein expression, whether they are functional in the small intestine has not been determined. Importantly, the distribution of cell types in the small intestine vs. the caecum or colon will dictate the cytokine milieu in the area that ultimately is the cause for inflammation. As discussed in chapter 5, how similar the cytokine profile in *ship*^{-/-} small intestines is to those seen in UC or CD is still largely unknown.

LPS in the *ship*^{-/-} intestine obviously does play a role in the inflammatory response since both heat-killed *Salmonella* and *Citrobacter* as well as LPS induced inflammation. However, whether the degree of inflammation seen in the intestine is dependent on the dose of LPS remains unknown. Identifying a low

dose of LPS that would lessen the degree of inflammation in *ship*^{-/-} ilea, or not induce the response altogether, would suggest that the reason microflora do not cause inflammation in the *ship*^{-/-} mouse is because they do not shed enough LPS to generate a response. Indeed the number of microflora in the small intestine, around 10⁴ bacteria, is many logs lower than the 10¹⁴ organisms that reside in the colon (Duchmann *et al.*, 1995). But, this fact again raises the question as to why LPS from microflora in the colon would not generate an inflammatory response in *ship*^{-/-} mice. Once again, the population of cells in the area and cytokine milieu most likely has a large impact on the outcome of intestinal pathology.

Finally, it is important to question the impact intestinal inflammation has on the development of systemic bacterial infections. While in the case of *Citrobacter* infection, small intestinal inflammation does not seem to affect the ability of bacteria to colonize the colon, *Salmonella* was found to replicate to high numbers in systemic sites and cause mortality in *ship*^{-/-} mice. A major virulence strategy for *Salmonella* is to breach the epithelial barrier in the intestine to gain access to underlying immune cells. In inflamed ilea of the *ship*^{-/-} mouse, more *Salmonella* may have access to the lamina propria, since there is extensive breakdown of the epithelial layer. In addition, the massive infiltration of immune cells to the submucosa provides ample opportunity for the bacterium to establish intracellular infection and traffic to systemic sites. Furthermore, the fact that inflammatory cytokine levels, besides TNF α , were not high in inflamed *ship*^{-/-} ilea may indicate that the innate cells present, such as macrophages, would not be activated against invading *Salmonella*. As such, the phenotype of these macrophages most likely is a strong determining factor in how efficient *Salmonella* is at establishing infection.

6.5 Future directions

Because SHIP is such a pluripotent regulator of both innate and adaptive immune cell signaling, it is most likely a critical component of gut and systemic immune responses to *Salmonella* and LPS during infection. How then can such

a complex mouse model be dissected to provide a mechanism by which SHIP determines susceptibility to *Salmonella*? In order to try to answer what the role of SHIP may be during systemic infection, *in vivo* studies using *Salmonella* mutants as well as infections of *ship/RAG*^{-/-} mice would be very effective. For example, SPI-2 deficient *Salmonella* mutants do not establish infection in systemic organs. Infecting *ship*^{-/-} mice with these bacteria, and assessing susceptibility to disease as well as bacterial load in various organs, would highlight whether cells involved in spreading *Salmonella* throughout the body are impaired in the *ship*^{-/-} mouse. In addition, using individual SPI-2 effector mutants may elucidate specific mechanisms by which SHIP could be targeted by *Salmonella* during *in vivo* infections. Furthermore, studies where wild type *S. Typhimurium* and SPI-2 mutants are tracked via immunofluorescence to see which cell types they may replicate within, and to what degree, would aid in our understanding of how SHIP deficiency effects the spread of systemic bacteria.

Combining SHIP deficiency and a lack of T and B cells, as in a *ship/RAG*^{-/-} mouse, would determine whether increased susceptibility to *Salmonella* infection in the *ship*^{-/-} mouse is dependent on adaptive immune responses. This would be an interesting model to study the outcome of both systemic replication of *Salmonella* as well as gut inflammation, since it is known that T and B cells play distinct roles in clearing *Salmonella* infections from systemic sites as well as the development of IBD. Once it is determined whether functional SHIP is required for adaptive cells to respond to *Salmonella* infection, it would be best to specifically inhibit the function of SHIP in single immune cell populations. This method has been extremely useful in determining the role of SHIP in regulating T cell responses. For example, T cell specific deletion of SHIP, using a Cre-lox targeting system, showed that T cell development, activation and phenotype are independent of SHIP expression, but Th2 bias requires functional SHIP (Tarasenko *et al.*, 2007). This work highlighted the fact that skewing of T cell populations towards a regulatory phenotype and elevated activation states of T cells in *ship*^{-/-} mice is not due to a lack of SHIP in the cells as originally thought, but rather due to the cytokine environment most likely created by hyper-

responsive innate cells. Especially useful to further this work, would be to target SHIP deletion in macrophages and, if possible in M2 macrophages, and to examine the effects on both gut inflammation and systemic Salmonellosis.

Finally, it would be extremely interesting to test the outcome of infectious diseases in a model where SHIP signaling was enhanced. Exploring this possibility has become realistic since a naturally occurring small molecule, called pelorol, can be used to activate SHIP *in vivo* (Yang *et al.*, 2005). More importantly, synthetic analogs of pelorol not only activate SHIP *in vivo*, but are also effective in preventing sepsis as well as various inflammatory disorders such as arthritis, dextran-sulfate induced colitis and cutaneous inflammation (Ong *et al.*, 2007). Therefore, would these SHIP activators be able to reduce susceptibility to *Salmonella* infection in susceptible mouse strains? If so, SHIP may be targeted as a therapy for systemic infections.

Of course, many other questions remain about the *ship*^{-/-} mouse and how it responds to disease. Since *ship*^{-/-} mice are highly susceptible to *Salmonella* but not *Citrobacter* infection, it would seem that SHIP deficiency may only affect responses to intracellular or systemic pathogens; however, other infection models must be established to more fully answer this question. Furthermore, would there be a difference in response to parasitic vs. bacterial infections, since the M2 macrophage population may skew T helper responses to a Th2 phenotype? Also, what is the mechanism behind the strong inflammatory response seen in the guts of *ship*^{-/-} mice and is this inflammation useful in studying IBD? The fact that so many questions remain, highlights the importance SHIP has in immunity and the potential to study other negative regulators and their roles not just in signaling, but also in determining the outcome of disease.

6.6 Significance

Salmonella infections are a significant threat to human health worldwide. While vaccinations and some antibiotic treatments can be effective in curbing disease, there are still millions of people that suffer severe illness or death from both typhoid and NTS. Research has provided a wealth of insight into the nature

of this pathogen, however it is clear that the immune response to *Salmonella* infection is extremely complicated. Because negative regulation of innate and adaptive responses to *Salmonella* plays an important role in the outcome of disease, examining how negative regulators like SHIP impact enteric disease can shed light on how better to control these infections. In fact, it is already being proposed that targeting negative regulation of immune responses by SHIP may be an effective means of providing therapy for inflammatory disorders such as arthritis and colitis, suggesting that negative regulator modulatory therapies may also be designed to combat infectious diseases. In a time when multifaceted anti-*Salmonella* vaccines are difficult to design and there is increased risk of antibiotic resistant *Salmonella* strains, the need for new infectious disease treatments is essential. In addition, effective therapies that dampen immune responses in the gut are necessary for the treatment of inflammatory bowel diseases (IBD) like Crohn's Disease (CD) and Ulcerative Colitis (UC) that are becoming increasingly common. This work shows that SHIP does play an important role in immune responses to both enteric pathogens as well as regulating gut immune responses, and could be explored as a potential target for both infectious diseases and IBD.

6.7 Concluding remarks

The *ship*^{-/-} mouse is a complex model to use in studying *Salmonella*, or any infections. Inherently, these mice have severe perturbations of many immune cell types and responses that would render them vulnerable to disease. Therefore, it is not surprising that the *ship*^{-/-} mouse is highly susceptible to *Salmonella* infection. However, what is interesting, is that SHIP deficiency does not impact the immune response to all pathogens in the same way and that it clearly has a major role in maintaining tolerance in the gut to microbial products. Furthermore, these studies suggest that macrophage heterogeneity plays an important role in *Salmonella* pathogenesis.

Clearly this work has just begun to probe how SHIP may be involved in immune responses to pathogens, but it underscores the importance of yet

another negative regulator in determining the outcome of disease. In addition, it has provided opportunities for future work to be done that may elucidate specific mechanisms by which SHIP regulates susceptibility to infection or how pathogens like *Salmonella* may have evolved to interact with negative regulators in the immune system. Most importantly, it has increased our knowledge of Salmonellosis and given insight as to how targeting negative regulators like SHIP could be explored as potential therapy for disease.

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Appendix 1: Thesis Material Included in Publications

Chapters 3 and 4 include material from the following manuscript, published in *Infection and Immunity* in May, 2008.

Jennifer L. Bishop, Laura M. Sly, Gerald Krystal, and B. Brett Finlay (2008). The inositol phosphatase SHIP controls *Salmonella* Typhimurium infection *in vivo*. *Infect. Immun.*, **76**:2913-22.

Chapter 5 includes material from a manuscript in preparation entitled "A role for SHIP in the small intestinal inflammatory response to enteric bacteria".

Appendix: 2 Contribution of Others

Chapter 3

I designed and performed all experiments in this chapter. Dr. Gerald Krystal kindly provided *ship*^{+/-} mice used for breeding *ship*^{-/-} and *ship*^{+/+} mice used in experiments. Dr. Laura Sly provided technical assistance for mouse genotyping. Drs. Guntram Grassl and Erin Boyle provided technical assistance for immunofluorescence and immunohistochemistry.

Chapter 4

I designed and performed all experiments in this chapter. Dr. Laura Sly provided technical assistance and guidance for the derivation of BMDM under M1 vs. M2 inducing conditions.

Appendix 3: Publications Arising from Graduate Work

Peer-reviewed articles

Bishop, J.L., L.M. Sly, G. Krystal, and B.B. Finlay. 21 April 2008. The inositol phosphatase SHIP controls *Salmonella* Typhimurium infection *in vivo*. Infect. Immun. doi:10.1128/IAI.01596-07.

Boyle, E.C.*, **J.L. Bishop***, G.A. Grassl* and B.B. Finlay (2007). *Salmonella*: from pathogenesis to therapeutics. J.Bacteriology, 189(5):1489-95.

Bishop, J.L.*, E.C. Boyle* and B.B. Finlay (2007). Bacterial cell wall modification as a means of surviving and evading the host innate immune response. Proc. Natl. Acad. Sci., 104(3): 691-2.

Coombes, B.K., M.J. Lowden, **J.L. Bishop**, M.E. Wickham, N.F. Brown, N. Duong, S. Osborne, O. Gal-Mor, B. Brett Finlay (2006). SseL is a novel *Salmonella*-specific translocated effector integrated into the SsrB-controlled type III secretion system. Infect. Immun., 75(2):574-80.

Bishop, J.L. and B.B. Finlay (2006). Friend or Foe? Antimicrobial peptides trigger pathogen virulence. Trends Mol. Med. 12(1):3-6.

**These authors contributed equally to this work.*

Abstracts

Bishop, J.L., L.M. Sly, G. Krystal and B.B. Finlay. SHIP and the M2 macrophage control susceptibility to *Salmonella* Typhimurium infection *in vivo* (2008). Keystone Symposia on Innate Immune Signaling, Keystone, CO.

Guarna, M., N. Glavas, H. Yang, A. Wang, A. Thompson, E. Dullaghan, N. Mookherjee, **J.L. Bishop**, O. Donini, M. Scott, M. Gold, B.B. Finlay, R. Hancock, J. North (2007). The Macrophage: Homeostasis, Immunoregulation and Disease. Keystone Symposia on Immunology, Copper Mountain, CO.

Coombes B.K., M.J. Lowden, **J.L. Bishop**, M.E. Wickham, N.F. Brown, N. Duong, S. Osborne, O. Gal-Mor, B.B. Finlay (2006). SseL is a novel *Salmonella*-specific translocated effector integrated into the SsrB-controlled SPI2 type III secretion system. Interscience conference on Antimicrobial Agents and Chemotherapy, Annual Meeting, San Francisco, CA.

Bishop, J.L., L.M. Sly, G. Krystal and B.B. Finlay (2005). Characterizing the role of the inositol phosphatase SHIP-1 in the innate immune response to *Salmonella* infection. Society for Leukocyte Biology 38th Annual Meeting, Oxford, UK.

Appendix 4: Animal Ethical Approvals

<https://rise.ubc.ca/rise/Doc/0/35FHAEI43M6KT2DF01603A0D43/f...>



THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE

Application Number: A05-1082

Investigator or Course Director: [Brett B. Finlay](#)

Department: Michael Smith Laboratories

Animals:

Mice C57/BL6 408
Mice Transgenic mice - various 147
Mice BALB/c 160
Mice C3H/He 114
Mice nramp +/- 40
Mice CD1 286
Mice 129/Sv 40
Mice SHIP +/- 43

Start Date: October 1, 2005

Approval Date: September 17, 2007

Funding Sources:

Funding Agency: Canadian Institutes of Health Research (CIHR)
Funding Title: Novel therapeutics that boost innate immunity to treat infectious disease

Funding Agency: The Foundation for the National Institutes of Health
Funding Title: Novel therapeutics that boost innate immunity to treat infectious diseases

Funding Agency: The Foundation for the National Institutes of Health
Funding Title: Novel therapeutics that boost innate immunity to treat infectious diseases

Funding Agency: Genome British Columbia

Funding Title: The Pathogenomics of Innate Immunity (PI2)

Funding Agency: The Foundation for the National Institutes of Health

Funding Title: Novel therapeutics that boost innate immunity to treat infectious diseases

Unfunded title: N/A

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

Office of Research Services and Administration
102, 6190 Agronomy Road, Vancouver, BC V6T 1Z3
Phone: 604-827-5111 Fax: 604-822-5093

Appendix 5: Pathology Scoring Worksheet

Sample ID _____

Tissue _____

Lumen	Empty		0	
	Necrotic epithelial cells	Scant	1	
		Moderate	2	
		Dense	3	
	Neutrophils	Scant	2	
		Moderate	3	
		Dense	4	
				Total
Surface Epithelium	No pathological changes		0	
	Regenerative change	Mild (<20%)	1	
		Moderate	2	
		Severe	3	
	Desquamation	Patchy(<30%)	1	
		Diffuse	2	
	Neutrophils in epithelium		1	
	Ulceration		1	
				Total
Mucosa	No pathological changes		0	
	Crypt abscesses	Rare (<15%)	1	
		Moderate (15-50%)	2	
		Abundant (>50%)	3	
	Mucinous plugs		1	
	Granulation tissue		1	
				Total
Submucosa	No pathological changes		0	
	Monocytes	1 aggregate	0	
		>1 small aggregate	1	
		Large aggregate	2	
	PMNs	None	0	
		Single PMNs	1	
		Aggregates	2	
	Edema	Mild (<10%)	0	
		Moderate (10-80%)	1	
		Severe (>80%)	2	
				Total
TOTAL SCORE=				