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2011

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Toll-like Receptor Signaling and its Effects on Microbial Pathogenesis

by

Nicholas Arpaia

A dissertation submitted in partial satisfaction of the requirements for the

degree of

Doctor of Philosophy

in

Molecular and Cell Biology

in the

Graduate Division

of the University of California, Berkeley

Committee in charge:

Professor Gregory M. Barton, Chair

Professor Mark S. Schlissel

Professor Qing Zhong

Professor Lee W. Riley

Fall 2011



Abstract

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By

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Doctor of Philosophy in Molecular and Cell Biology

University of California, Berkeley

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Toll-like receptors (TLRs) contribute to host resistance to microbial pathogens and drive the evolution of virulence mechanisms. We have examined the relationship between host resistance and pathogen virulence using mice with a functional allele of the Nramp-1 gene and lacking combinations of TLRs. Mice deficient in both TLR2 and TLR4 were highly susceptible to the intracellular bacterial pathogen *Salmonella typhimurium*, consistent with reduced innate immune function. However, mice lacking additional TLRs involved in *S. typhimurium* recognition were less susceptible to infection. In these TLR-deficient cells, bacteria failed to upregulate *Salmonella* pathogenicity island 2 (SPI-2) genes and did not form a replicative compartment. We demonstrate that TLR signaling enhances the rate of acidification of the *Salmonella* containing phagosome, and inhibition of this acidification prevents SPI-2 induction. Our results indicate that *S. typhimurium* requires cues from the innate immune system to regulate virulence genes necessary for intracellular survival, growth, and systemic infection.

I would like to dedicate this work to my father,  
Nicola Arpaia—your perseverance has always been an inspiration.

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## Acknowledgements

First and foremost, I would like to thank my mentor, Gregory M. Barton, for making my graduate experience incredible. Without your motivational wisdom none of this would have ever been possible. You have shaped me into a scientist that values hard work, thoughtful inquisition, and taking time to reflect through celebration. For the long conversations about science and life that we've had—those that I remember, and the ones I never will—thank you for supporting me. I can finally say without hesitation, “I'm aware.”

It's impossible to accurately express in words the extreme gratitude that I have for my lab mates, the ‘OG’ crew—Maria Mouchess, Roman Barbalat, Laura Lau and Sarah Ewald. Maria, *mi hermana de otra mama*, thank you for teaching me Spanish and for chocottone. Roman, you will always be my baymate—forever, no exceptions, so too bad—it was fun. Laura (*Lauz*) don't tell Roman, but you're my favorite baymate—thank you for long walks to the eucalyptus grove, dried mango and a constant stock of life's nectar.

To other members of the lab that joined later—thank you for indulging me. Bettina, you fed me, walked me halfway home, and answered all of my questions. Alex, your constant contributions to my life soundtrack have kept me going. Kelsey, I've said it before, in a different context, but you're my rock—thanks. Jernej, you made being a graduate student much more enjoyable day by day. Allison, before you, I didn't think I was capable of mentoring a graduate student. Zaqisha, don't forget, you're me after I leave—I have complete faith you'll be great. Meghan, just when I thought the lab had reached its coolness asymptote, you arrived. You gave *Lauz* a run for her money, but you understand why she won favorite baymate. *Bling*, you're an incredible scientist. I was honored to share a bench with you—keep at it. Thank you for helping me with experiments. You've kept me sane these last few months.

Chris Rae, Laura Shield, Richard Kline and Henry Jones—you were perfect housemates. Chris Rae, thank you for teaching me how to surf, walking to school with me, long talks about everything, and being my best friend—‘shred’ is a noun, a verb, and a way of life. Laura Shield, thank you holding my hand through waxing and tattoos, you're my sister. Richard Kline, thank you for eating my leftovers and letting me eat yours.

My incredible friends, the people that make me feel comfortable being me—thank you. Ian Schneider, you are the only person who ever told me to “give [myself] credit.” Justin (JC) Lee, we teach every city we visit how it's done—where to next? Adrian Anchondo, my last roommate—you feed the cat too much. Please stop. Kris Kersey, I admire you. You are responsible for my take-no-prisoners attitude. John Bishop, for always making sure I took time away from work to enjoy the city. Pam Barone, you have been my friend for nearly 20 years—here's to 20 more.

To my thesis committee, Mark Schlissel, Lee Riley, and Qing Zhong. I appreciate all of your support and your signing of this dissertation.



To members of the fourth floor of LSA, and Vance and Portnoy labs, I appreciate all of the helpful discussions. Russell, thank you for being a supportive second PI.

Most importantly, I would like to thank my family—Nicolina, Gennaro, *Nonno* Gennaro, *Nonna* Antonietta Arpaia and *Nonno* Armando and *Nonna* Caterina Lisi.

Dad, this thesis was already dedicated to you so I won't say more—you're amazing.

Mom, thank you so much. I can honestly say I would never have made it without you.

Thank you for picking up the phone and listening to me, even when I forgot you were in a different time zone. Your conversations were refreshing and necessary.

Gennaro (Je), thank you for being the only one that was on my side when no one else was, for writing me letters from wherever you were, for making things harder on me when they seemed like they couldn't get any worse, and for keeping some secrets and putting others on blast. Most of all, thank you for being the most incredible brother that a kid could ever have. I'm always so proud of you.

*Nonni, voi siete sempre stati la mia forza—grazie.*

### **Abbreviations List**

BMM- Bone Marrow-derived Macrophage  
CFU- Colony Forming Units  
CHX- Cycloheximide  
DC- Dendritic Cell  
HEK- Human Embryonic Kidney  
IFN- Interferon  
IP- Intraperitoneal  
LD- Lethal Dose  
MHC- Major Histocompatibility Complex  
MOI- Multiplicity of Infection  
OG- Original Gangsta  
*S.t.- Salmonella typhimurium*  
SCV- *Salmonella* Containing Vacuole  
SPI- *Salmonella* Pathogenicity Island  
T3SS- Type 3 Secretion System  
TEM- Transmission Electron Microscopy  
TNF- Tumor Necrosis Factor

## **Chapter 1: An introduction to TLR signaling and detection of viral and bacterial pathogens**

*Portions of this chapter were previously published (Arpaia and Barton, 2011).*

### **TLR Signaling Pathways**

#### **Overview**

Toll-like receptors (TLRs) are an essential arm of the innate immune response to bacteria, viruses and fungi and link recognition of distinct features of these microbes to the induction of pro-inflammatory signaling pathways (Iwasaki and Medzhitov, 2010; Kawai and Akira, 2010). These receptors are able to respond to broad classes of pathogens because each TLR recognizes specific conserved microbial features. Multiple microbial products can serve as ligands, including LPS (TLR4), lipopeptides (TLR2/1 and TLR2/6), flagellin (TLR5), unmethylated CpG motifs in DNA (TLR9), and RNA (TLRs 3, 7 and 8).

TLRs consist of an extracellular ligand-binding domain, a transmembrane domain, and a cytosolic signaling domain called the Toll-interleukin 1 receptor homology domain (TIR) (Kawai and Akira, 2010). Signaling occurs via homotypic binding of TIR domains on the receptor with those on signaling adaptors. All TLRs, with the exception of TLR3, utilize the common signaling adaptor myeloid differentiation factor 88 (MyD88). MyD88 also contains a death domain that recruits IL-1R-associated kinase (IRAK) family members upon recruitment to the TLR TIR. IRAK proteins then dissociate from the MyD88/TLR complex and interact with tumor necrosis factor receptor-associated factor 6 (TRAF6) to mediate downstream signaling.

Both TLR3 and TLR4 are able to recruit a second adaptor, TIR-domain-containing adapter-inducing interferon- $\beta$  (TRIF). TRIF activation initiates a TRAF3-dependent signaling cascade that results in the dimerization and activation of TANK (TRAF family member-associated NF- $\kappa$ B activator)-binding kinase 1 (TBK1) and inhibitor of NF- $\kappa$ B kinase (IKKi). This TBK1/IKKi complex leads to phosphorylation of the transcription factor IRF3, allowing it to translocate into the nucleus. In macrophages and DCs, activation of either pathway leads to induction of signal transduction cascades culminating in activation of transcription factors (NF- $\kappa$ B, AP-1, IRFs) that coordinate expression of proinflammatory genes, such as TNF- $\alpha$ , type I interferons, IL-1, IL-12, and many others (discussed below).

Additional complexity arises from the fact that TLRs differ in their downstream signaling pathways as well as their localization within the cell. As alluded to above, only TLR3 and TLR4 can induce TRIF activation while all other TLRs only activate MyD88. These differences in signal transduction affect gene induction downstream of each TLR, although the relevance of differential TLR signaling to the immune response against infection remains poorly understood. TLRs can be further subdivided based on their localization within the cell. TLR1, TLR2, TLR4, TLR5, and TLR6 are localized at the cell surface, while TLR3, TLR7, TLR8, and TLR9 are localized within endosomes and lysosomes (Ewald et al., 2008b; Hacker et al., 1998; Latz et al., 2004; Leifer et al., 2004; Underhill et al., 1999). Recruitment of both types of TLRs to phagosomes has been reported (Latz et al., 2004; Underhill et al., 1999). The subcellular localization of TLRs correlates with the type of ligands recognized by each group. TLRs on the surface generally recognize components on the exterior of pathogens, while intracellular TLRs recognize nucleic acids. Presumably, microbes must be degraded before nucleic acids

become available for TLR binding. A final implication of intracellular localization of certain TLRs is that microbial ligands must be internalized before recognition by these TLRs can occur (Hacker et al., 1998). It is likely that this mechanism helps to limit recognition of self nucleic acids (Barbalat et al., 2011).

Another layer of specificity in TLR signaling is the control of type I IFN induction after stimulation. All TLRs implicated in viral recognition can induce expression of this family of cytokines (Stetson and Medzhitov, 2006). As mentioned above, TLR3 and TLR4 are able to activate the signaling adaptor TRIF, inducing IRF3 phosphorylation, translocation and the transcription of type I IFN genes. A second pathway for the induction of type I IFN involves the transcription factor IRF7 and takes place downstream of TLR7 and TLR9. Interestingly, this pathway functions primarily in a specialized subset of dendritic cells known as plasmacytoid dendritic cells (pDC) (Barbalat et al., 2011). The ability for these cells to uniquely respond to TLR7 and TLR9 ligands by making IFN has been attributed to their high expression of IRF7. However, by delivering TLR7 and TLR9 ligands to early endosomal compartments via lipid complexes, researchers have been able to activate type I IFNs in macrophages as well (Honda et al., 2005). These observations suggest that ligand trafficking may be the unique feature that allows IFN production within pDCs. Whether macrophages are able to make IFN via specialized ligand delivery *in vivo* is still unknown. However, the importance of this pathway in the recognition and control of viral replication has been repeatedly shown and will be discussed further.

Mice or cells lacking both MyD88 and TRIF are incapable of TLR signaling and have been used extensively to study the role of TLRs in immunity to many pathogens (Yamamoto et al., 2003). One caveat associated with these studies, however, is the fact that MyD88 is also required for signaling by the IL-1 receptor family (IL-1R, IL-18R, and IL-33R). Thus, phenotypes in MyD88xTrif-deficient mice cannot be unequivocally attributed to TLR-deficiency. This will be come an important aspect of our approach in later Chapters of this dissertation.

### ***Functional Consequences of TLR Signaling***

A specific feature of TLR responses that does not apply to cytosolic sensors is the detection of defective or dead microbes that do not cause active infection but still activate TLRs on the surface and within the phagosome. This also applies to the engulfment of apoptotic-infected cells. The detection of these microbial products after engulfment highlights the phagosome-autonomous functions of TLR signaling, outlined in Figure 1.1. As mentioned, TLR3, TLR7 and TLR9 reside within the phagosome and TLR4 has been shown to be internalized with ligand upon activation (Kagan et al., 2008). Once activated, these TLRs induce more rapid acidification of the phagosome, presumably through the recruitment of the V<sub>1</sub> sector subunits A and E (of the Vacuolar-ATPase) to the phagosomal membrane (Arpaia et al., 2011; Blander and Medzhitov, 2004; Blander and Medzhitov, 2006b; Trombetta et al., 2003). The release of reactive oxygen and nitrogen species by recruitment of NADPH oxidase or mitochondria to the phagosome is another hallmark of phagosome-autonomous TLR-mediated maturation (Lambeth, 2004; Underhill and Ozinsky, 2002; West et al., 2011). Cationic antimicrobial peptides are also secreted into the phagosome in response to TLR signaling.

Along with proteases that are active at the low pH of the mature phagosome, microbial proteins are degraded into peptides, loaded into MHC class II and shuttled to the surface of the cell. Further, antigens present within this mature phagosome can be loaded into MHC class I for activation of cytotoxic CD8 T cells via a process known as cross presentation (Fonteneau et al., 2003; Katunuma et al., 1994; Shen et al., 2004). The precise details of the cross presentation pathway are still elusive, however, it is widely accepted that engulfed, infected dead or dying cells activate TLRs and that their microbial antigens are capable of being presented in class I MHC after engulfment by CD8 $\alpha^+$  dendritic cells (Schulz et al., 2005).

TLR signaling also leads to the upregulation of costimulatory molecules on the surface of the signaling cell. These include CD80, CD86 and CD40 (Figure 1.1). These molecules are required along with antigen presentation for the activation of T lymphocytes. Class II MHC molecules presenting microbial antigens are also upregulated on the cells surface. These molecules, upregulated via TLR signaling, serve to activate T cells for protective immunity.

As discussed previously, the intracellular nucleic acid sensing TLRs are capable of inducing type I IFN in pDCs. The overall importance of this cytokine is exemplified by the extreme susceptibility of type I IFN receptor mice to infections (Stetson and Medzhitov, 2006). Signaling via the type I IFN receptor leads to the upregulation of many genes that serve the function of shutting off host translation and inducing apoptosis, thus rendering the cell inhospitable for viral replication. This cytokine is also critically important for other immune responses. For example, IFN can lead to the differentiation and activation of natural killer (NK) cells. These cells serve the function of killing virally infected cells that try to evade antigen presentation via MHC down-regulation (Biron and Brossay, 2001; Biron et al., 1989; Delale et al., 2005). Type I IFN has also been shown to induce cross presentation by dendritic cells and leads to the upregulation of MHC Class I, further increasing the activation of CD8 T cells.

Other pro-inflammatory cytokines that are produced downstream of TLRs include IL-6, IL-12 and TNF- $\alpha$ . These cytokines serve numerous functions including promoting the survival and proliferation of B and T cells, activating NK cells, and inducing local inflammation to recruit other cells to sites of infection

## **TLRs and Viruses**

Nucleic-acid sensing plays an important role in recognizing viral genomes. This requirement for sensing nucleic acid is due, in part, to limited viral ‘foreignness.’ Viruses utilize host machinery and nucleotides to assemble into infectious particles, and the innate immune system relies on ligand delivery of viral nucleic acid to intracellular compartments to distinguish viral ligands from self. Although there are examples of recognition of viral envelope proteins by TLRs, I will first discuss nucleic acid sensing and touch upon these other forms of recognition later.

### ***Recognition of DNA viruses by TLR9***

Unmethylated CpG motifs within bacterial nucleic acid were the first ligands identified to activate TLR9 (Hemmi et al., 2000). Since then, many DNA viruses have also been shown to activate this TLR, including those from the herpesvirus (Asselin-Paturel et al., 2001; Dalod et al., 2003; Feldman et al., 1994; Fiola et al., 2010; Krug et

al., 2004b; Lund et al., 2003; Lund et al., 2006; Rölle and Olweus, 2009; Sato and Iwasaki, 2004; Zucchini et al., 2008), adenovirus(Zhu et al., 2007), poxvirus(Samuelsson et al., 2008) and torquetenovirus (TTV) families(Rocchi et al., 2009). Both  $\alpha$ - and  $\beta$ -herpesviruses have genomes that are rich in CpG motifs. Herpes simplex viruses (HSV) 1 and 2 have been shown to activate TLR9 in spleen and bone marrow cultures to produce pro-inflammatory cytokines and type I IFN (Krug et al., 2004b; Lund et al., 2003), and similar results have also been observed with mouse cytomegalovirus (MCMV) (Delale et al., 2005; Krug et al., 2004a; Zucchini et al., 2008). Although the contribution of TLR9 recognition of these viruses has clearly been reported in vitro, the phenotype of TLR9-deficient mice in vivo does not completely phenocopy that of mice deficient in the signaling adaptor MyD88. These results suggested that other members of the TLR/IL-1R family might play a redundant role in recognition of these viruses, and it was later shown that the remaining MyD88-dependent recognition may be mediated by detection of viral envelope proteins by TLR2 and possibly RNA intermediates produced during viral replication by TLR3 and TLR7 (discussed later) (Fiola et al., 2010; Sato et al., 2006; Zucchini et al., 2008).

It is noteworthy that very few examples exist of viruses suppressing CpG motifs within their genomes as a strategy to evade nucleic acid detection. This lack of evidence can perhaps be explained by the constraint on mutability of compact viral genomes with few non-coding sequences. One example of CpG suppression has recently been reported for a member of the  $\gamma$ -herpesvirus family, murine herpesvirus 68 (MHV68) (Pezda et al., 2011). In this study it was reported that CpG suppression, most likely mediated by cytosine to thymine conversion, within the genome of MHV68, allows the virus to evade TLR9 detection and largely avoid detection in vivo. Further, during latent stages of infection, CpG motifs within the viral genome were found to be methylated, and therefore unable to stimulate TLR9. These alterations in genome composition lead to reduced type I IFN and pro-inflammatory cytokine production. Nevertheless, it is difficult to prove that TLR9 recognition was the selective pressure leading to suppression of these motifs.

### ***Recognition of ssRNA by TLR7/8***

The first ligands identified to stimulate TLRs 7 and 8 were members of the imidazoquinolones family which share structural similarity with ribonucleosides (Hemmi et al., 2002). The imidazoquinolones were long known to stimulate a potent antiviral response, and it wasn't until the discovery of these TLRs that the receptors mediating this response were appreciated. The precise structural motifs or ssRNA sequences (analogous to CpG motifs for TLR9 stimulation) that are capable of stimulating TLR7 are not precisely known. G-U rich and even PolyU RNA are capable of stimulating TLR7 (Diebold et al., 2004). Single stranded RNA is extremely labile, and host-derived RNA rarely comes into contact with intracellular TLRs because it is rapidly broken down by extracellular RNases. In contrast, viral particles protect viral RNA from degradation; destruction of viral particles within phagosomes releases viral RNA for recognition by TLRs 7 and 8.

Similarly to TLR9, TLRs 7 and 8 are capable of inducing type I IFNs in pDCs via the signaling adaptor MyD88. HIV, influenza and vesicular stomatitis virus (VSV) have all been reported to stimulate TLR7(Diebold et al., 2004; Heil et al., 2004; Lund et al.,

2004). The genomic RNA of influenza virus is capable of inducing IFN $\alpha$  from mouse dendritic cells in a TLR7 dependent manner, and similar results were obtained when comparing stimulations of wildtype and TLR7-deficient cells with the U5 region of HIV-1 RNA.

### ***Recognition of dsRNA by TLR3***

TLR3 recognizes dsRNA as well as the synthetic analog, polyI:C (Alexopoulou et al., 2001; Bell et al., 2006; Bell et al., 2005; Choe et al., 2005). This receptor signals through TRIF and leads to production of IFN in all cell types in which it is expressed. Despite the discovery that TLR3 is a potent inducer of IFN in response to dsRNA, *in vivo* evidence that TLR3 deficiency results in exacerbated disease in response to viral infection was lacking for many years—leading some to believe that TLR3 may not actually be a receptor of viral dsRNA directly (Edelmann et al., 2004).

It has since been appreciated, however, that TLR3 may play a role in recognition of dsRNA intermediates within engulfed apoptotic cells that have been infected with ssRNA viruses (Schulz et al., 2005). The viruses used in this study were encephalomyocarditis virus and Semliki Forest virus, both of which are ssRNA viruses, lending to the possibility that dsRNA, produced during the replication of these viruses, is detected by TLR3 within the apoptotic cells after phagocytosis.

Interestingly, several reports have also shown that in models of West Nile virus and Influenza A virus (both ssRNA viruses), pro-inflammatory cytokines produced in a TLR3-dependent manner can actually lead to increased viral pathology (Daffis et al., 2008; Hidaka et al., 2006; Le Goffic et al., 2006; Le Goffic et al., 2007; Wang et al., 2004). These reports further confirm the notion that dsRNA intermediates are likely generated and detected *in vivo*. Adding further complexity to how TLR3 may participate in viral recognition, TLR3-deficient mice are more susceptible to the DNA virus MCMV (Tabeta et al., 2004). Also, human patients with deficiencies in TLR3 or signaling molecules downstream of this receptor show predisposition to HSV-induced encephalitis (Audry et al., 2011; Casrouge et al., 2006; Guo et al., 2011; Pérez de Diego et al., 2010; Zhang et al., 2007). This suggests that dsRNA may be produced by the secondary structures of viral transcripts derived from the viral genome of DNA viruses.

### ***Non-nucleic acid recognition of Viral ligands by TLRs***

As mentioned earlier, the recognition of viral nucleic acid by TLRs may be the most reliable method of detecting viruses because of ligand delivery, the constraints of mutating viral genomic contents, and the fact that the viral particle is assembled using host machinery. Despite these conceptual considerations, there are a handful of examples of viral proteins serving as TLR ligands. In principle, the sequence or structure of these detected proteins must be constrained for this strategy to work. Mutating the regions that are detected by TLRs should make the viral particle non-functional; otherwise, the virus would easily be able to mutate these proteins to avoid detection. The clear exception to this idea is when TLR-mediated inflammation is important for viral pathology, as is supported in some infection models discussed below.

HSV-1, HSV-2, HCMV, MCMV, VV, respiratory syncytial virus (RSV) and the hemagglutinin protein of measles virus have all been shown to activate TLR2 (Barbalat et al., 2009; Bieback et al., 2002; Compton et al., 2003; Kurt-Jones et al., 2004; Murawski

et al., 2009; Sato et al., 2006; Szomolanyi-Tsuda et al., 2006). In response to VV infection, TLR2 on inflammatory monocytes is capable of inducing type I IFN, and depletion of these cells leads to elevated levels of Vaccinia virus in ovaries of mice (Barbalat et al., 2009). TLR2 signaling has also been shown to be important in CD8 T cells where it is critical for clonal expansion and memory formation following VV infection (Quigley et al., 2009). These examples suggest that TLR2-mediated detection of VV is important for the host to be able to generate a protective response against the virus.

In a very different example, TLR2 activation by certain isolates of HSV-1 seems to induce excessive inflammation. In this model, TLR2-deficient animals are somewhat protected from HSV-induced encephalitis (Kurt-Jones et al., 2004). The responding cells in this HSV example may be generating a pro-inflammatory response without the production of protective IFN, aberrantly leading to increased encephalitis pathology.

Mouse mammary tumor virus (MMTV) and RSV have both been implicated to activate TLR4 (Burzyn et al., 2004; Jude et al., 2003; Kurt-Jones et al., 2000; Rassa et al., 2002; Tal et al., 2004). In the case of MMTV, TLR4 leads to the production of IL-10, an anti-inflammatory cytokine, and allows for the virus to enter a stage of persistence by dampening the immune response against it (Jude et al., 2003). On the other hand, the role of TLR4 detection of RSV seems to be protective. TLR4-deficient mice have reduced IL-6 production in response to RSV and infants with polymorphisms in TLR4 are at higher risk for severe RSV infection (Kurt-Jones et al., 2000; Tal et al., 2004).

### **TLRs and Pathogenic Bacteria: *Salmonella typhimurium***

Many products of bacteria are capable of activating TLRs. These include flagellin (TLR5), LPS of gram-negatives (TLR4), bacterial lipoproteins of gram-negative and positives (TLR2), and genomic DNA and RNA (TLR9 and TLR7, respectively). As is the case for viral pathogens, both live and dead bacteria (pathogenic and non-pathogenic) activate TLRs. Pathogenic bacteria, however, have strategies for evading the consequences of TLR activation. The most extreme examples are intracellular pathogens that reside within the phagosome. These bacteria have elaborate virulence mechanisms for transforming the phagosome into a more hospitable replicative compartment. In discussing bacterial recognition by TLRs, I will focus on the lifecycle and virulence of a model pathogen, *Salmonella typhimurium*. Analysis of this host-microbe interaction provides a clear example of how bacteria have evolved to survive in the face of TLR activation—and as will be discussed later, require this activation as a contextual cue to initiate virulence.

### ***S. typhimurium* Lifecycle and Virulence**

*Salmonella enterica* serovar *typhimurium* (*S. typhimurium*) is an intracellular, gram-negative bacterium that can survive and replicate within host macrophages. This survival occurs despite the recognition of bacteria by multiple TLRs (Figure 1.2). Similar to viruses, activation of TLRs can lead to bacterial killing through induction of reactive oxygen, reactive nitrogen, phagolysosomal fusion, and other antimicrobial mechanisms. *S. typhimurium* can survive within macrophages, however, by disrupting phagosome maturation, by inhibiting the innate immune response, and by creating an environment suitable for replication. This sophisticated strategy requires that *S. typhimurium* recognize its presence in the phagosome and coordinate expression of the many bacterial virulence



genes necessary for intracellular survival. This strategy of innate immune evasion through coordinate expression of virulence genes is a common theme within intracellular pathogenic bacteria. Thus, studying the interaction of *S. typhimurium* with the innate immune response gives a good overview of TLR interactions with bacterial pathogens.

The importance of TLRs in immunity to *S. typhimurium* has been demonstrated using cells or mice deficient in TLRs or TLR signaling components (Royle et al., 2003; Takeuchi et al., 1999; Weiss et al., 2004). In general, cells or mice lacking TLR function are more susceptible to *S. typhimurium* infection than wildtype controls (Weiss et al., 2004). A caveat of these studies, however, is that they have almost all been performed with mice on the C57BL/6 background. C57BL/6 mice are highly susceptible to *S. typhimurium* due to a non-functional allele of the *Nramp-1* gene (*Nramp-1<sup>S/S</sup>*) (Govoni et al., 1996; Vidal et al., 1995). This caveat will become an important consideration in the experiments that will be discussed later in this dissertation.

*S. typhimurium* is the causative agent of human gastroenteritis, also referred to as salmonellosis. *S. typhimurium* is the number one cause of bacterial foodborne-disease outbreaks, with over 16,000 reported cases and 20 deaths from 585 outbreaks between 1998-2002 (Pegues et al., 2005). Human-adapted serovars of *S. typhimurium*, such as *Salmonella typhi*, can cause a severe and systemic infection known as typhoid fever. *S. typhimurium* causes a similar typhoid-like disease in mice, and much of our knowledge of *Salmonella* pathogenesis in humans has come from mouse infection studies using *S. typhimurium*.

Both serovars of *Salmonella* enter the host through the gut via ingestion of contaminated food. Once there, *Salmonella* colonizes and penetrates the intestinal epithelium by utilizing a set of genes (encoded within *Salmonella* pathogenicity island 1, SPI-1) to actively induce its own entry into intestinal epithelial cells (Carter and Collins, 1974; Hohmann et al., 1978). *S. typhimurium* preferentially interacts with Peyer's patches, invading and destroying specialized M-cells before spreading to the adjacent epithelium (Jones et al., 1994). In the former case, SPI-1 utilizes a type III secretion system (T3SS) that translocates numerous bacterial effectors into the host cell that induce rapid polymerization of host actin, leading to bacterial uptake (Altmeyer et al., 1993; Bliska et al., 1993; Fu and Galán, 1999; Galán and Curtiss, 1989; Galán et al., 1992; Ginocchio et al., 1994; Hardt et al., 1998; Hayward and Koronakis, 1999; Pace et al., 1993). After crossing the epithelial barrier, *S. typhimurium* is phagocytosed by macrophages residing along the basement membrane that likely deliver bacteria to systemic sites of infection (Clark et al., 1996; Everest et al., 1999; Vazquez-Torres and Fang, 2000). CD18-positive monocytes that sample gut contents and phagocytose bacteria provide *S. typhimurium* another route of passage through the intestinal epithelium. These cells have been implicated in bacterial dissemination and the elicitation of adaptive immune responses (Vazquez-Torres et al., 1999).

Survival within macrophages requires a distinct set of genes, many of which are encoded within a different pathogenicity island, termed SPI-2. SPI-2 also encodes a T3SS, and expression is induced after the bacterium is phagocytosed by the host cell. SPI-2 effectors allow *S. typhimurium* to transform the phagosome into a replicative niche—hijacking a normally degradative compartment to create the *S. typhimurium* containing vacuole (SCV). *S. typhimurium* is able to replicate and survive within the SCV until it reaches sufficient numbers, exits the cell, and spreads to surrounding cells and

tissues. Studies in which *S. typhimurium* lacking SPI-2 function were injected intraperitoneally (IP) (bypassing the requirement for SPI-1) highlight the importance of SPI-2 for systemic infection. SPI-2 mutants are avirulent *in vivo*, and are unable to survive within macrophages in *in vitro* assays (Chakravorty et al., 2002; Cirillo et al., 1998; Hensel et al., 1997; Hensel et al., 1998).

Several key two-component regulatory systems have been implicated in regulation of genes required for intracellular survival. PhoP/PhoQ controls expression of genes involved in lipid A modifications, increased resistance to antimicrobial peptides, and many other properties required for intracellular survival (Belden and Miller, 1994; Castelli et al., 2000; García Vescovi et al., 1996; Groisman et al., 1989; Gunn and Miller, 1996; Miller et al., 1989). The PhoQ sensor kinase is repressed by divalent cations, such as  $Mg^{2+}$ ,  $Mn^{2+}$ , and  $Ca^{2+}$ , and activated by cationic antimicrobial peptides (CAMP) and acidic pH (Prost and Miller, 2008). Although a decrease in divalent cations in the phagosome could theoretically activate PhoP/PhoQ, it is generally believed that CAMP and low pH are more likely triggers, especially because  $Ca^{2+}$  and  $Mg^{2+}$  concentrations in the phagosome do not appear to reach low enough concentrations to activate PhoQ (Prost and Miller, 2008).

Whether PhoP/PhoQ activation leads to direct induction of the SPI-2 TTSS is controversial, but two other two-component systems are clearly involved. EnvZ-OmpR modulates gene expression in response to osmotic conditions. In the absence of OmpR, *S. typhimurium* is unable to replicate inside macrophages. It is believed that the function of this two-component system is to regulate expression of virulence genes, including another two-component system, SsrA-SsrB, implicated in regulation of SPI-2 genes (Kim and Falkow, 2004). SsrA-SsrB regulates the expression of genes encoding the SPI-2 TTSS and effectors that collectively lead to formation of the SCV and ultimately allow *S. typhimurium* to replicate within macrophages (Deiwick et al., 1999; Garmendia et al., 2003; Hensel et al., 1998; Ochman et al., 1996; Rappl et al., 2003; Shea et al., 1996). The coordinate expression and activation of these two-component systems signals *S. typhimurium* to initiate virulence gene programs. Therefore, it is not surprising that disruption of or failure to activate either of these systems leads to the inability to survive within macrophages and drastically decreases virulence (Hensel et al., 1998; Miller et al., 1989; Ochman et al., 1996).

Although these two-components systems have been widely studied, it remains to be determined what ligand(s) within the phagosome are detected by the sensor-kinase. *S. typhimurium* has evolved these complex regulatory mechanisms to distinguish its location within the cell and activate gene programs to survive in these compartments. Host proteins that generate the unique phagosomal environment that is sensed via these two-component systems are therefore just as instrumental in initiating the virulence-signaling cascade.

### ***Host Genes Involved in Restricting S. typhimurium Replication***

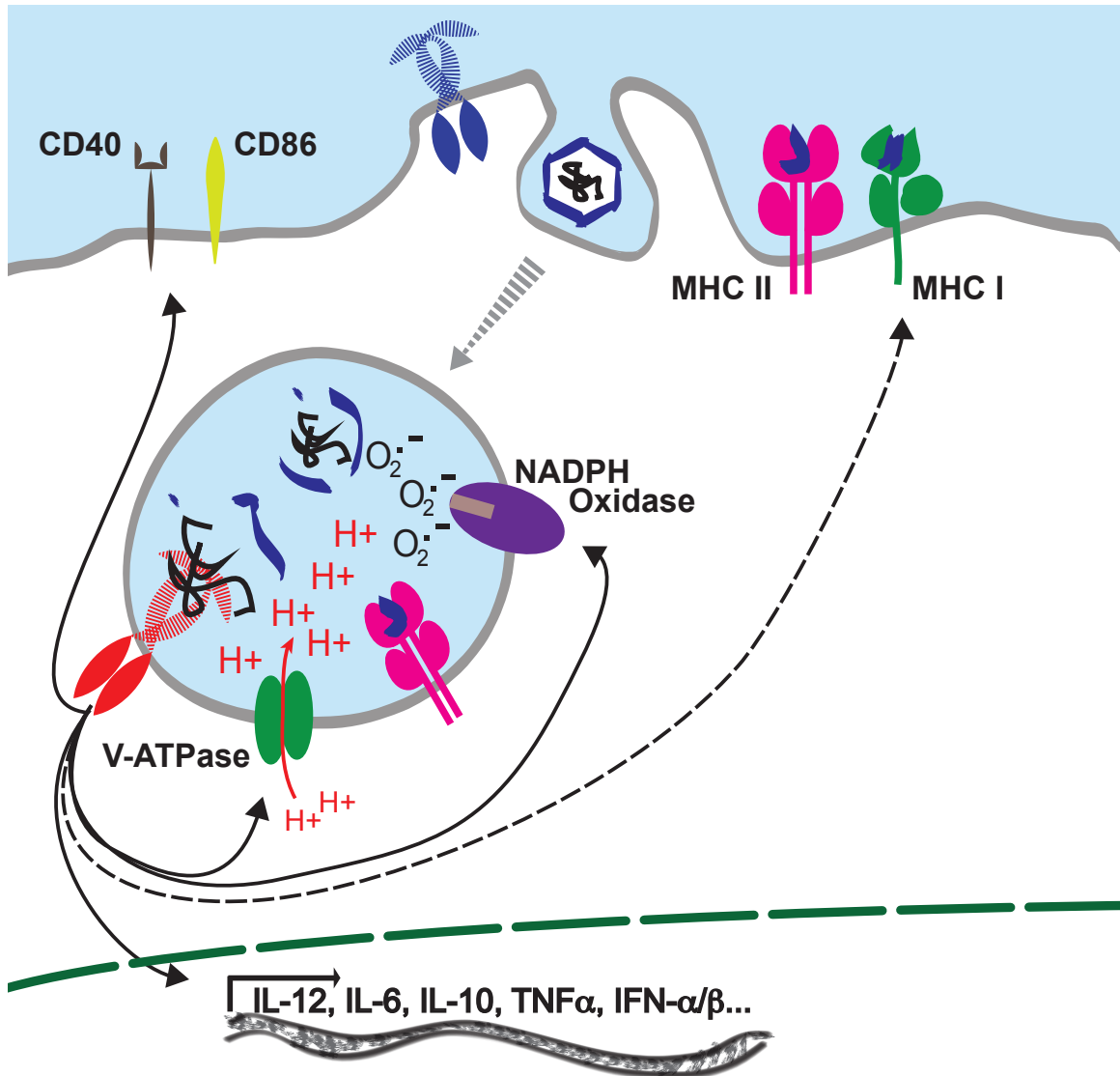
While *S. typhimurium* pathogenesis enables the bacterium to circumvent many aspects of innate immunity, a number of host genes do function to limit bacterial replication. The best evidence for the role of certain genes in immunity to *S. typhimurium* comes from analysis of mice with targeted deletions or naturally occurring mutations in these genes. These types of studies have demonstrated an important role for TLRs in the

control of *S. typhimurium* replication (Kobayashi et al., 2002; Royle et al., 2003; Takeuchi et al., 1999; Weiss et al., 2004). Mice lacking TLR4, both TLR2 and TLR4, or MyD88 are more susceptible to infection than wildtype controls (Weiss et al., 2004). In addition, macrophages derived from these mice are deficient in limiting intracellular *S. typhimurium* replication in vitro (Weiss et al., 2004).

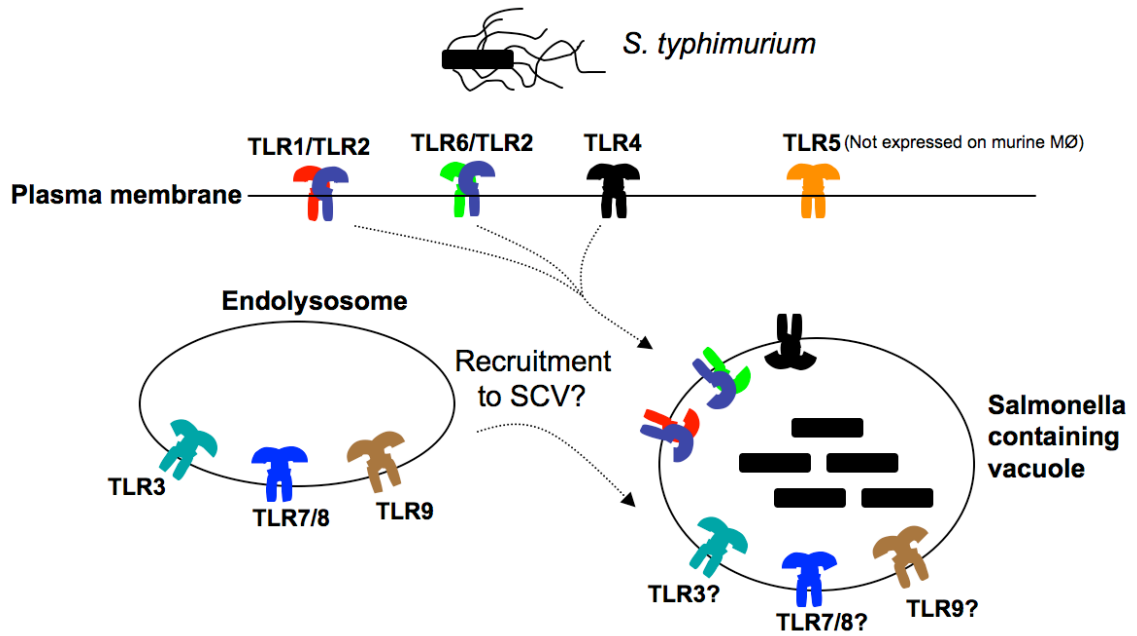
The mechanisms by which TLRs limit *S. typhimurium* replication have not been definitively worked out. As discussed above, TLR activation can lead to a number of antimicrobial mechanisms, including production of reactive oxygen and nitrogen, production of antimicrobial peptides, acidification of phagosomes, increased proteolytic activity, and enhanced phagolysosomal fusion. In certain instances, analysis of cells or mice lacking components required for these mechanisms have revealed their importance in the host response to *S. typhimurium*. For example, mice lacking NADPH oxidase or both NADPH oxidase and iNOS show increased susceptibility to *S. typhimurium* infection (Mastroeni et al., 2000; Shiloh et al., 1999; Vazquez-Torres et al., 2000a; Vazquez-Torres et al., 2000b).

A complicating factor in the analysis of host genes necessary for immunity to *S. typhimurium* is the fact that most studies use mice with a nonfunctional allele of the *Nramp-1* gene. *Nramp-1* encodes a 10-pass transmembrane protein that localizes to lysosomes and functions as a transporter of divalent cations. Many inbred mouse strains, including the commonly used C57Bl/6 strain, possess a *Nramp-1* allele with a point mutation that leads to a glycine to aspartate coding change at position 105, within the second transmembrane domain of the protein. Inbred strains with the Asp<sup>105</sup> allele are highly susceptible to a number of intracellular pathogens, including *S. typhimurium*, BCG, and Leishmania. For example, the oral LD<sub>50</sub> of *S. typhimurium* for C57Bl/6 (Asp<sup>105</sup> allele) mice is 4 x 10<sup>5</sup> bacteria, whereas the oral LD<sub>50</sub> for 129 (Gly<sup>105</sup> allele) is greater than 10<sup>9</sup> bacteria (Kim et al., 2003; Monack et al., 2004). Exactly how *Nramp-1* restricts bacterial growth remains an area of active study. Current models favor the idea that *Nramp-1* pumps Mn<sup>2+</sup> and possibly other ions out of the phagosome, which creates a nutrient poor environment for *S. typhimurium*. The precise details by which *Nramp-1* negatively impacts *S. typhimurium* intracellular replication are not directly relevant to this proposal. What is relevant, however, is the fact that the analyses of almost all other host genes have been carried out in genetic backgrounds in which *Nramp-1* is nonfunctional. Consequently, even the wildtype mice in these studies are extremely susceptible to infection, making it difficult to truly assess the role of TLRs in the immune response to *S. typhimurium*. The importance of this issue is further underscored by recent work suggesting that expression of functional *Nramp-1* in DCs and macrophages may lead to enhanced TLR signaling (Valdez et al., 2008; Valdez et al., 2009).

As will be discussed in this dissertation, we have generated individual and multiple TLR-deficient mice on an *Nramp-1*<sup>R/R</sup> background. These mice allow us to properly dissect the role of TLR signaling in *S. typhimurium* pathogenesis for the first time. In fact, our unique reagents have allowed us to make a very striking observation: namely that *S. typhimurium* requires TLR signaling in order to replicate intracellularly—this will be the focus of the remainder of this dissertation.



**Figure 1.1. Functional consequences of TLR signaling.** Engulfed microbes are degraded in endosomes and/or phagosomes. Released microbial nucleic acid stimulates intracellular nucleic acid-sensing TLRs (red) leading to phagosome autonomous responses including recruitment of V-ATPase (green) and NADPH oxidase (purple). Microbial antigens generated upon degradation of the virus are loaded into MHC class II and shuttled to the surface (pink). In certain cell types, antigens are loaded into MHC class I via cross presentation (dashed line, green). TLR activation also leads to the upregulation of costimulatory molecules, CD40 (brown) and CD86 (lime), as well as a signaling cascade that leads to the transcription of cytokine genes.



**Figure 1.2. Interaction of TLRs with *Salmonella*.** The combinations of TLRs implicated in *Salmonella* immunity are shown. Note the different localization of TLR1, 2, 4, 5, and 6 versus TLR3, 7, 8, and 9. The extent to which TLRs are present in and/or recruited to the SCV is not well understood.

## Chapter 2: *Salmonella typhimurium* requires TLR signaling for replication and in vivo pathogenesis

*Portions of this chapter were previously published* (Arpaia et al., 2011).

### Background

The mammalian immune system has evolved under selective pressures imposed by infectious microbes. In early stages of infection the innate immune system is particularly essential for limiting microbial replication and spread before the adaptive response is mounted. Accordingly, pathogens have evolved virulence strategies to block or circumvent aspects of innate immune function leading to increased pathogen burden and reduced host fitness. For intracellular pathogens, an additional virulence mechanism is the ability to survive and replicate within host cells. The interplay between host innate immune function and pathogen virulence mechanisms largely determines the ultimate outcome of most infections, so these host-pathogen interactions are predicted to select for the emergence of antagonistic traits (Hedrick, 2004; Rausher, 2001; Woolhouse et al., 2002).

Innate immune receptors detect infection by recognizing conserved microbial features common to broad classes of microbes (Janeway, 1989; Medzhitov, 2007). The Toll-like receptors (TLRs) target a range of microbial ligands, including lipopolysaccharide (TLR4), lipoproteins (TLR2), flagellin (TLR5), unmethylated CpG motifs in DNA (TLR9), double-stranded RNA (TLR3), and single-stranded RNA (TLR7 and TLR8) (Akira et al., 2001; Kawai and Akira, 2005). Expression of TLRs on innate immune cells links microbial recognition to induction of antimicrobial mechanisms, such as production of reactive oxygen and nitrogen species and expression of antimicrobial peptides, which confer host resistance to infection. In addition, TLR activation can promote adaptive immunity through control of dendritic cell (DC) maturation (Iwasaki and Medzhitov, 2004).

To study the antagonism between innate immunity and pathogen virulence, we have focused on TLR-mediated recognition of *Salmonella enterica* serovar *typhimurium*. *S. typhimurium* is a gram-negative bacterium that can survive and replicate within host macrophages (Coburn et al., 2007). Survival within macrophages requires a distinct set of genes, many of which are encoded within *Salmonella* pathogenicity island 2 (SPI-2) (Galan, 2001; Shea et al., 1996; Waterman and Holden, 2003). SPI-2 encodes a type 3 secretion system (T3SS) that is expressed after the bacterium is phagocytosed by the host cell (Cirillo et al., 1998; Pfeifer et al., 1999; Valdivia and Falkow, 1997). Translocation of SPI-2 effectors into the host cell transforms the phagosome into a compartment that supports bacterial replication, the *Salmonella* containing vacuole (SCV) (Marcus et al., 2000). Multiple signals have been implicated in the transcriptional induction of SPI-2, including cation deprivation, phosphate starvation, and low pH (Chakravorty et al., 2005; Cirillo et al., 1998; Deiwick et al., 1999; Kim and Falkow, 2004; Rappl et al., 2003). Most of the studies implicating these signals have been performed on bacteria grown *in vitro*; whether the same signals are responsible for induction of SPI-2 genes within the phagosome remains controversial. In addition to the transcriptional regulation of SPI-2 genes, the assembly and stability of the T3SS apparatus appears to depend on acidic pH (Rappl et al., 2003; Yu et al., 2010). Thus, the use of host-derived contextual cues plays a prominent role in the lifecycle of *Salmonella*.

TLRs are critical for host defense against *S. typhimurium*. Recognition is largely mediated by TLR4, although TLR2 and TLR5 also contribute (Feuillet et al., 2006; Hapfelmeier et al., 2005; O'Brien et al., 1980; Royle et al., 2003; Smith et al., 2003; Uematsu et al., 2006; Vazquez-Torres et al., 2004). To overcome this redundancy, investigators have used mice lacking the common TLR adaptor MyD88 or lacking both MyD88 and TRIF (Hapfelmeier et al., 2005; Weiss et al., 2004). Consistent with selective pressure imposed by host resistance, *S. typhimurium* has evolved mechanisms to subvert TLR-mediated recognition or to avoid the consequences of TLR activation. For example, modification of lipid A reduces recognition by TLR4 and increases resistance to antimicrobial peptides (Detweiler et al., 2003; Guo et al., 1997; Guo et al., 1998). Bacterial mutants unable to make these modifications are severely attenuated (Miller et al., 1989). In addition, *S. typhimurium* replication is enhanced in a macrophage cell line previously activated by TLR ligands, although neither the mechanism behind this observation nor its relevance to pathogenesis are known (Wong et al., 2009). Altogether, these examples suggest that TLR-mediated innate recognition has driven the evolution of antagonistic traits that enable the survival of *S. typhimurium* within macrophages.

While the aforementioned studies clearly underscore the close relationship between host innate immunity and pathogen virulence strategies, two potential caveats encouraged us to revisit these questions. First, although MyD88-KO and MyD88xTRIF-KO mice are susceptible to many pathogens including *S. typhimurium*, this hypersusceptibility cannot be unequivocally attributed to TLRs, as MyD88 is also required for signaling by members of the IL-1 receptor (IL-1R) family, and mice deficient in IL-1R are more susceptible to infection (Mayer-Barber et al., 2010; Raupach et al., 2006). Second, most studies examining the importance of TLRs in *S. typhimurium* immunity have used mice or cells with a nonfunctional allele of the *nramp-1* gene. *Nramp-1* encodes a multipass transmembrane protein that localizes to lysosomes and functions as a transporter of divalent cations (Forbes and Gros, 2001). Many inbred mouse strains, including the commonly used C57Bl/6 strain, possess a *nramp-1* allele with a point mutation that leads to a glycine to aspartate coding change at position 169, within the second transmembrane domain of the protein (Govoni et al., 1996; Vidal et al., 1995; Vidal et al., 1996). Inbred strains with the Asp<sup>169</sup> allele are extremely susceptible to a number of intracellular pathogens, including *S. typhimurium* (Vidal et al., 1995; Vidal et al., 1993; Vidal et al., 1996). Nramp-1 also plays an important role in human cells, as individuals with polymorphisms in *nramp1* are more susceptible to several intracellular pathogens, including *Mycobacterium tuberculosis* (Malik et al., 2005). We reasoned that the contribution of other innate immune components may be masked in studies with mice that are already quite susceptible due to nonfunctional Nramp-1. To avoid these caveats in our studies of host-pathogen interactions, we generated mice with a functional allele of *nramp1* that lack individual or multiple TLRs. Analysis of *S. typhimurium* pathogenesis in these mice led to a striking finding. While mice lacking a subset of the TLRs involved in *S. typhimurium* recognition showed increased susceptibility to infection, a lack of additional TLRs resulted in reduced susceptibility. Importantly, this phenotype required functional Nramp1. The loss of virulence correlated with an inability of bacteria to survive and replicate within macrophages.

## Materials and Methods

### Cell Culture

HEK293 cells (ATCC) were cultured in DMEM supplemented with 10% (vol/vol) FCS (Hyclone), L-glutamine, penicillin-streptomycin, sodium pyruvate and HEPES, pH 7.2. All supplements and base-media were purchased from Gibco/Invitrogen, unless otherwise noted. For generation of bone marrow derived macrophages (BMMs), bone marrow isolated from femurs and tibia was cultured in BMM media (RPMI-1640 supplemented with 10% (vol/vol) fetal calf serum, L-glutamine, penicillin-streptomycin, sodium pyruvate, HEPES, pH 7.2, and M-CSF containing supernatant) for 5 days as previously described (Ewald et al., 2008a). Day 5 BMMs were harvested by scraping and plated in RPMI-1640 medium (as above, but without the addition of M-CSF and penicillin-streptomycin) for infection the following day. In some cases, BMMs were frozen in 95% FCS, 5% DMSO and stored at -150°C for later use. For thawing BMM stocks, cells were plated in BMM media, expanded for 2 additional days, and then plated for experiments as described above.

### Bacterial Strains and Infections

*S. typhimurium* cultures were inoculated from single colonies and grown shaking at 250 rpm overnight in LB (Fisher Scientific) supplemented with 200 µg/mL streptomycin (in the case of SL1344, Invitrogen) at 37°C. The following morning, a 1:10 dilution of the quantified culture (in PBS, Gibco) was shaken in 25% normal mouse serum (Jackson ImmunoResearch) for 25 minutes, washed twice in PBS, and added to antibiotic free RPMI-1640 culture medium (above) for infection at the indicated MOIs. Cells were spin-infected for 5 minutes at 750 rpm, then incubated at 37°C for 25 minutes. Following incubation, cells were washed twice with PBS before the addition of 10µg/mL gentamicin-containing media. For MOIs greater than 10, cells were first incubated in 100µg/mL gentamicin-containing media for 1h, followed by a reduction to 10µg/mL gentamicin for the remainder of the experiment. For intracellular CFU determination, cells were washed once with PBS at the indicated time post-infection and lysed in 1% Triton-X 100 in PBS for 5 minutes at 37°C. Lysates were diluted to 0.2% Triton-X 100 with antibiotic-free complete RPMI, and dilutions were plated on LB agar plates containing 200 µg/mL streptomycin for colony enumeration. *PipB2-2xHA* (12032) and wildtype 12032 were kind gifts from S. Meresse. LT2 and SL1344, were obtained from S. Falkow (Stanford University).  $\Delta$ *fliB/fliC* (LT2) was a gift from R. Vance (UC Berkeley). *L. monocytogenes* was a gift from D. Portnoy (UC Berkeley). Constitutive SPI-2,  $\Delta$ *hha* (SL1344), was constructed by recombination using the  $\lambda$  Red recombinase method (Datsenko and Wanner, 2000) and phage transduction. For *L. monocytogenes* infections, BMMs were plated onto coverslips overnight and infected the following morning at an MOI of 1. Intracellular growth was monitored by gentamicin intracellular replication assay, as described (Leber et al., 2008). For *L. pneumophila* infections, BMMs were infected at an MOI of 0.05 with a *L. pneumophila* ( $\Delta$ *fla*) strain expressing the *lux* operon. Growth was monitored by increase in luminescence over time, as described (Lightfield et al., 2008).



### ***TLR Signaling in HEK293 Cells***

All HEK293 cell lines (with the exception of TLR4/MD2/CD14) expressing the indicated TLRs were made by stably transfecting each TLR into a HEK293 cell line that stably expresses an ELAM NF $\kappa$ B-luciferase reporter. For the TLR4/MD2/CD14 HEK293 line, HEK293 cells stably expressing TLR4/MD2 were transiently transfected by Lipofectamine LTX (Invitrogen) lipofection with CD14 and the ELAM NF $\kappa$ B-luciferase reporter the night before the assay. Heat-killed *S. typhimurium* (generated by incubation at 60°C for 30 minutes) was used to stimulate each of the cell lines at the indicated relative MOI (considering one doubling event from plating the night before). For measurement of TLR9 responses, HEK293 cells stably expressing a TLR9/TLR4 chimeric protein were stimulated with *S. typhimurium* genomic DNA purified by phenol:chloroform extraction. This chimeric receptor is surface localized, bypassing the need for ligand internalization (Barton et al., 2006). For all assays, cells were stimulated for 8h, lysed in passive lysis buffer (Promega), and luciferase activity was quantified using an LMaxII-384 luminometer (Molecular Devices). Data are shown as fold luminescence over unstimulated cells. The following TLR ligands were purchased from Invivogen: BLP (Pam<sub>3</sub>SK<sub>4</sub>), R848, Flagellin, and LPS. Phosphorothioate CpG oligonucleotides (5'-TCCATGACGTTCCCTGACGTT-3') were purchased from Integrated DNA Technologies.

### ***Measurement of Cell Death***

For lactate dehydrogenase (LDH) release assays, cells were plated in 96-well plates in non-antibiotic phenol-free DMEM (Gibco) supplemented as above. At the indicated timepoints post-infection, plates were spun at 1800 rpm for 5 minutes, and supernatants were taken for LDH quantification using the CytoTox 96 Non-radioactive cytotoxicity kit (Promega). Percent death was calculated as the amount of LDH released from infected cells minus untreated cells, divided by the amount of LDH released from cells lysed with 1% Triton-X 100. For Annexin V staining, BMMs were infected (as above) with an MOI of 10 and harvested and stained at 8 hours post-infection with Annexin V-FITC (BD Pharmingen) in Annexin V staining buffer and analyzed on an FC500 flow cytometer (Beckman Coulter).

### ***Measurement of BMM activation***

To measure nitric oxide production, BMMs were treated overnight with 100 $\mu$ g/mL recombinant IFN $\gamma$  (R&D Systems). The next day, BMMs were infected with bacteria (as described above) or stimulated with LPS (Invivogen) or phosphorothioate CpG oligonucleotide (5'-TCCATGACGTTCCCTGACGTT-3') (Integrated DNA Technologies). Nitric oxide production was quantified in cellular supernatants by Griess assay (all reagents from Sigma Aldrich) (Vazquez-Torres et al., 2008).

For measurement of TNF production, BMMs were infected with bacteria or treated with TLR ligands (as described above). 30 min later, BMMs were treated with brefeldinA (eBiosciences). After an additional 7.5h, cells were harvested, fixed, permeabilized, stained with anti-TNF antibody (eBioscience), and analyzed on an FC500 flow cytometer (Beckman Coulter). Fixation and permeabilization was performed according to manufacturer's instructions (eBioscience). For infections, all steps prior to fixation were performed in the presence of 10 $\mu$ g/mL gentamicin. For bafilomycinA1

pretreatment, BMMs were pretreated for 2h prior to infection/stimulation with 100  $\mu$ M bafilomycinA1 (EMD/Calbiochem), and treated as above with harvesting at 6h post-infection.

### ***Mice and in vivo Infections***

All animal experiments were carried out under approval from the animal care and use committee (ACUC) of the University of California. TLR2-, TLR4-, TLR9-, MyD88- and TRIF-deficient mice were generated and kindly provided by S. Akira (Osaka University). Mice were intercrossed to generate strains lacking multiple genes. All strains were backcrossed onto the C57Bl/6 background while maintaining the functional *Nramp1* allele ( $G^{169}$ ). The degree of backcrossing was verified by SNP analysis comparing 129S1 to C57Bl/6. Briefly, genomic DNA isolated from tails of backcrossed mice was purified for Illumina-based 129S1 vs. C57Bl/6 SNP detection performed by the Harvard-Partners Center for Genetics and Genomics (HPCGG). A total of 510 SNPs across the genome showed greater than 90% C57Bl/6 character as indicated by at least one allele of the C57Bl/6 SNP at each locus tested. The remaining 129S1 SNPs were within regions adjacent to loci of targeted genes (and therefore unlikely to be lost through backcrossing without a rare crossover event).

For survival and CFU enumeration experiments, age-matched mice were transferred to new cages and withheld food 14 hours before infection. Animals were inoculated by gavage with 100  $\mu$ L of the indicated CFU of *S. typhimurium* (SL1344) or *Yersinia enterocolitica* (8081) in PBS, then given food and water *ad libitum*. For CFU enumeration, organs were harvested and homogenized in PBS using a Polytron PT2100 homogenizer at 17000 rpm (Kinematica), serial diluted, and plated on 200  $\mu$ g/mL streptomycin (for *Salmonella*) or 1  $\mu$ g/mL irgasan-containing (for *Yersinia*) LB-agar plates.

## Results

### ***Multiple TLRs are involved in recognition of S. typhimurium***

Like many bacterial pathogens, *S. typhimurium* possesses ligands for multiple TLRs, yet assessing the relative importance of individual TLRs for protective immunity is complicated by numerous factors, including masking of ligands by the pathogen, abundance of the ligands, as well as potential redundancy among TLRs. As an initial approach toward identifying which TLRs were relevant for innate recognition of *S. typhimurium*, we utilized HEK293 reporter cell lines stably expressing an NF- $\kappa$ B-luciferase reporter construct. Stimulation of these cells with heat-killed bacteria resulted in robust induction of NF- $\kappa$ B, which we attributed to endogenous TLR5 expressed by these cells (Figure 2.1A). Accordingly, this response was abrogated when cells were stimulated with bacteria lacking flagellin (*S.t.  $\Delta$ fliB/fliC*). To measure activation of other TLR family members, HEK293 reporter cells stably expressing individual TLRs were stimulated with bacteria lacking flagellin (to eliminate the contribution of endogenous TLR5). Using this approach, we were able to observe activation of TLR2 and TLR4 by *S. typhimurium* (Figure 2.1A). Furthermore, *S. typhimurium* genomic DNA was capable of activating a surface localized version of TLR9 (Figure 2.1A).

While these results confirmed that TLR2, TLR4, TLR5, and TLR9 may play a role in recognition of *S. typhimurium*, they relied on ectopic receptor expression and did not address the relative importance of each TLR during infection. To this end, we infected bone marrow-derived macrophages (BMMs) lacking combinations of TLRs with *S. typhimurium* and measured production of nitric oxide (NO). In agreement with previously published studies, BMMs lacking both TLR2 and TLR4 (TLR2x4-KO) produced much less NO than wildtype BMMs (Figure 2.1B). The remaining response was partially dependent on TLR9, as BMMs lacking TLR2, TLR4, and TLR9 (TLR2x4x9-KO) produced even less NO than TLR2x4-KO cells. Similar results were observed when tumor necrosis factor alpha (TNF) production was measured (Figure 2.1C). Importantly, all genotypes of BMMs responded equivalently to the TLR7 ligand, R848, indicating that the cells were otherwise equivalent (Figure 2.2). The small amount of TNF and NO produced in TLR2x4x9-KO BMMs was dependent on other TLRs, as BMMs lacking both MyD88 and TRIF (and therefore all TLR-dependent signaling) did not respond to *S. typhimurium* (Figure 2.1B & C). As TLR5 is not expressed in murine BMMs, we reasoned that the residual TNF and NO produced by TLR2x4x9-KO BMMs was most likely due to TLR7 or TLR3 signaling. To address this possibility directly, we pretreated TLR2x4x9-KO BMMs with bafilomycinA1, an inhibitor of the vacuolar ATPase (V-ATPase) that prevents activation of endosomal TLRs. BafilomycinA1 treatment inhibited TNF production in TLR2x4-KO and TLR2x4x9-KO BMMs to almost background levels, suggesting that TLR7 and/or TLR3 are responsible for the remaining TNF production in response to *S. typhimurium* (Figure 2.1D).

Collectively, these data indicate that TLR2, TLR4, TLR9, and TLR7 (and/or TLR3) each contribute to the recognition of *S. typhimurium* in infected BMMs. While the role for TLR2 and TLR4 is well established, the contribution of nucleic acid sensing TLRs is somewhat surprising. TLR9 and TLR7 have been implicated in recognition of bacterial DNA and RNA, respectively (Bafica et al., 2005; Mancuso et al., 2009), although the role of this recognition in the host response to *S. typhimurium* is not known.

Their role in *S. typhimurium* recognition by BMMs suggests that exposure of nucleic acid ligands (either through secretion or release after lysis) occurs during infection.

### ***TLR signaling is required for in vivo pathogenesis***

Having established that multiple TLRs recognize and respond to ligands derived from *S. typhimurium* in BMMs *in vitro*, we sought to test the effect this signaling would have on *in vivo* pathogenesis of the bacteria. As discussed in the Introduction, *in vivo* studies of *S. typhimurium* pathogenesis in mice are complicated by the presence of an inactivating polymorphism of the *nramp1* gene present in commonly used inbred strains, including C57BL/6 (Vidal et al., 1995; Vidal et al., 1993; Vidal et al., 1996). Because this hypersusceptibility may mask the role of other innate immune genes, we crossed a functional allele of the *nramp1* gene (Gly<sup>169</sup>) onto the C57BL/6 background and generated TLR-deficient or TLR adaptor-deficient mice with functional Nramp1. To confirm that all strains were similarly backcrossed onto C57BL/6 we used an Illumina platform-based SNP analysis (510 SNPs across the mouse genome). All the strains used in this study were at least 90% C57BL/6 (see Materials and Methods).

Based on the many studies examining the importance of TLRs in various infection models, we expected that reduced TLR function would lead to greater susceptibility to infection. Indeed, all TLR2x4-KO mice died within 16 days when challenged orally with *S. typhimurium*, while 75% of the wildtype mice survived for the duration of the experiment (Figure 2.3A). By contrast, TLR2x4x9-KO mice were *less* susceptible to infection than TLR2x4-KO mice, despite a greater impairment in TLR function (Figure 2.3A). This increased survival was not a consequence of reduced immunopathology and elevated tolerance due to reduced TLR function (Schneider and Ayres, 2008). In fact, TLR2x4x9-KO mice had lower numbers of bacteria 4 days post-infection in spleens, livers, ceca and mesenteric lymph nodes (MLNs) relative to TLR2x4-KO mice (Figure 2.3B). Thus, despite less robust innate immune function, *S. typhimurium* was less virulent in TLR2x4x9-KO mice.

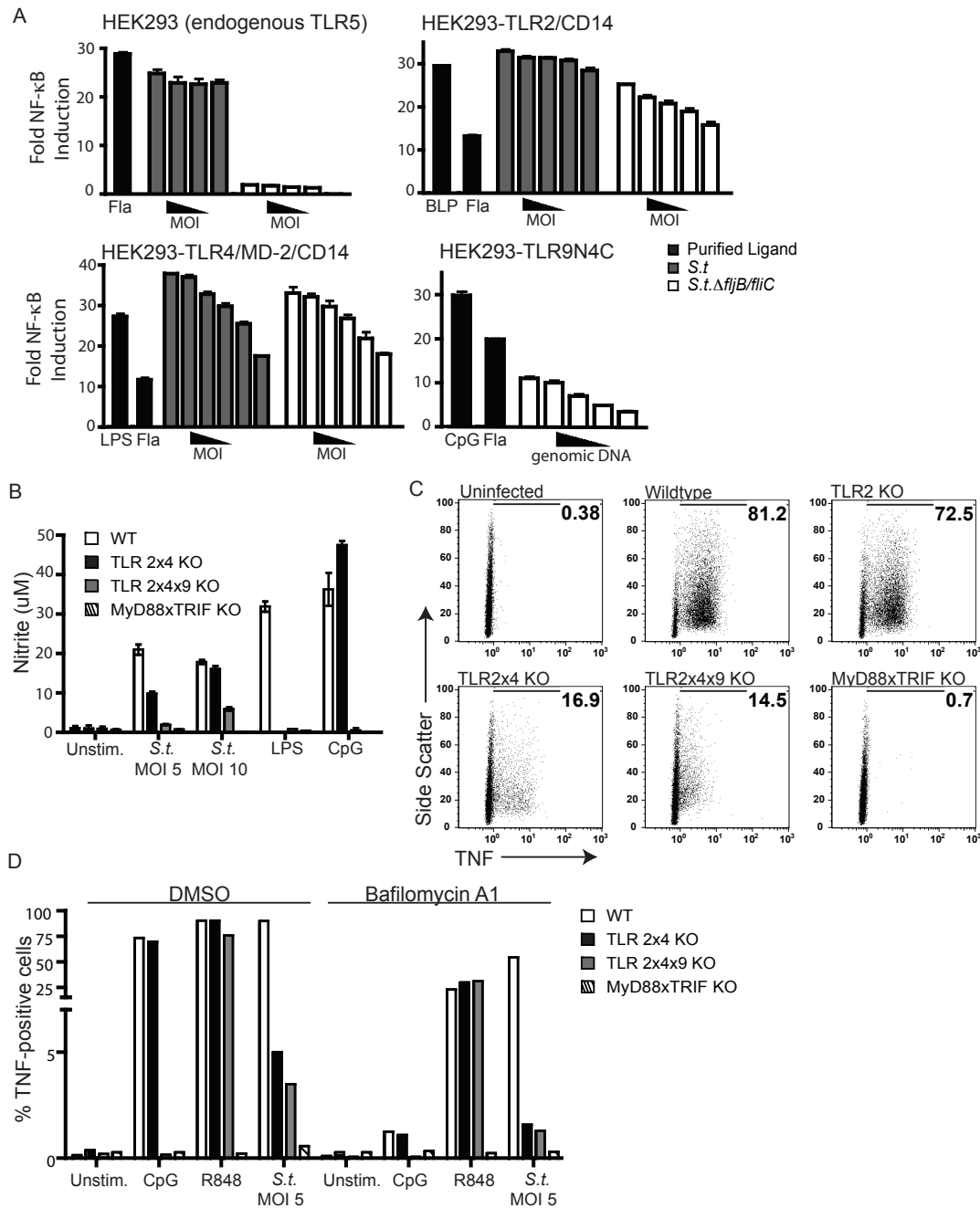
The difference in susceptibility between TLR2x4-KO and TLR2x4x9-KO mice can be interpreted in two different ways: either TLR9 plays a negative role in immunity to *S. typhimurium*, or TLR signaling in general is required for *S. typhimurium* virulence. To distinguish between these two possibilities, we tested the susceptibility of mice lacking TLR4 and TLR9 (TLR4x9-KO) to *S. typhimurium* infection. We hypothesized that if TLR9 were playing a negative role in immunity, then any genotype lacking TLR9 would be resistant to infection. Instead, TLR4x9-KO mice were as susceptible to infection as TLR2x4-KO mice, indicating that lack of TLR9 by itself does not confer increased resistance to infection (Figure 2.3A). Thus, the data presented suggest that overall TLR signaling is in some way required for *S. typhimurium* virulence. Despite this apparent requirement, MyD88-KO and MyD88xTRIF-KO mice (with wildtype Nramp1) were highly susceptible to *S. typhimurium* infection (Figure 2.4). The extreme sensitivity of these mice relative to TLR2x4x9-KO mice is most likely due to the role of MyD88 downstream of the IL-1, IL-18, and IL-33 receptors, especially considering the increased susceptibility of mice deficient in these pathways (Mayer-Barber et al., 2010; Raupach et al., 2006). Thus, to examine the role for TLR signaling in *S. typhimurium* virulence we must use TLR-deficient mice, not mice lacking common signaling adaptors.

One potential caveat of these *in vivo* studies is that the commensal flora may be different between TLR2x4-KO and TLR2x4x9-KO mice. Indeed, a number of recent studies have reported alterations in commensal communities in mice lacking certain TLRs or TLR signaling adaptors (Vijay-Kumar et al., 2008; Wen et al., 2008). Such differences may influence the outcome of *S. typhimurium* infection, especially considering our use of the natural oral route of infection. To address this possibility directly, we challenged wildtype, TLR2x4-KO and TLR2x4x9-KO mice with a different gram-negative enteric pathogen, *Yersinia enterocolitica* (*Y. enterocolitica*). In contrast to our experiments with *S. typhimurium*, TLR2x4x9-KO mice were equally, if not more, susceptible relative to TLR2x4-KO mice when challenged orally with *Y. enterocolitica* (Figure 2.3C). *S. typhimurium* and *Y. enterocolitica* share a similar route of intestinal colonization, although their lifestyles after crossing the intestinal barrier are quite distinct, as *Y. enterocolitica* is primarily an extracellular pathogen. Importantly, the differential sensitivity of TLR2x4x9-KO mice to these two enteric bacteria argues that alterations in commensal flora are not contributing to the phenotypes of TLR2x4-KO and TLR2x4x9-KO mice. Instead, the reduction in TLR signaling in TLR2x4x9-KO mice appears to specifically impact the virulence of *S. typhimurium*.

### ***TLR signaling is required for intracellular growth of bacteria***

Because survival within macrophages is required for systemic infection (Fields et al., 1986; Leung and Finlay, 1991), we next examined survival and replication in BMMs lacking various TLRs. BMMs were infected with *S. typhimurium* for 30 minutes, followed by addition of gentamicin-containing media to kill any extracellular bacteria, and intracellular CFU were measured over time. Consistent with our *in vivo* experiments, *S. typhimurium* was able to replicate in TLR2x4-KO BMMs but not in TLR2x4x9-KO BMMs (Figure 2.5A). To ensure that the increase in CFU was due to intracellular growth, we counted bacteria in individual macrophages by immunofluorescence microscopy. The number of bacteria per cell in TLR2x4-KO BMMs accumulated over time, while the number of bacteria per cell in TLR2x4x9-KO BMMs remained constant, indicating that bacterial replication was responsible for the differences in CFU between genotypes (Figure 2.5B, C). We observed a similar lack of bacterial replication in MyD88xTRIF-KO BMMs (Figure 2.5A). Unlike our *in vivo* experiments, the phenotype of MyD88xTRIF-KO BMMs is most likely due to a deficiency in TLR signaling, as the IL-1 receptor family is not involved in the initial recognition of *S. typhimurium* within BMMs *in vitro*. Furthermore, TLR4x9-KO BMMs supported bacterial replication similarly to TLR2x4-KO BMMs, corroborating the conclusions from our *in vivo* experiments that the difference between TLR2x4-KO and TLR2x4x9-KO mice is not due to the specific lack of TLR9, but rather an overall reduction in TLR signaling (Figure 2.5B, C). We also ruled out the possibility that TLR2x4x9-KO and MyD88xTRIF-KO BMMs suffer from a global defect that prevents bacterial replication. Two other intracellular bacteria, *Listeria monocytogenes* and *Legionella pneumophila*, replicated equivalently in wildtype, TLR2x4-KO, TLR2x4x9-KO and MyD88xTRIF-KO BMMs (Figure 2.6). In addition, *Salmonella* replicated well in MyD88xTRIF-KO BMMs lacking Nramp1 (Figure 2.6). These results indicate that phagosomes of TLR-deficient cells are formally capable of supporting bacterial growth, but the combination of functional Nramp1 and lack of TLR signaling prevents *Salmonella* from replicating.

Collectively, these data suggest that *S. typhimurium* requires TLR signaling for replication in macrophages. However, the apparent lack of replication in wildtype BMMs would appear to contradict this conclusion, as TLR function is normal in these cells. When we examined the number of bacteria per cell by immunofluorescence microscopy, though, we observed an increase in bacteria per cell over time similar to what we observed in TLR2x4-KO BMMs (Figure 2.5C). Through the course of these analyses, we noticed that the number of wildtype BMMs decreased quite significantly over the course of the infection. This observation suggested that the lack of bacterial replication in wildtype cells was due to do greater amounts of host cell death which renders otherwise intracellular bacteria susceptible to killing by gentamicin. To test this possibility directly, we measured death of infected BMMs from each genotype using two assays: quantification of lactate dehydrogenase (LDH) and staining with annexinV. By these measures, wildtype BMMs exhibited greater cell death at 6 and 8 hours post-infection relative to each of the other genotypes (Figure 2.5D, E). As wildtype BMMs are the only genotype with functional TLR4, the increased death of these cells seems likely due to a previously described TLR4-dependent, TRIF-dependent cell death that occurs in *S. typhimurium* infected cells (Cook et al., 2007; Hsu et al., 2004; Park et al., 2002). Thus, the apparent lack of replication as measured by CFU in wildtype BMMs (Figure 2.5A) is the result of macrophage cell death followed by gentamicin-mediated killing of the bacteria. In contrast, the inability of *S. typhimurium* to replicate in TLR2x4x9-KO or MyD88xTRIF-KO BMMs is due to a different mechanism.

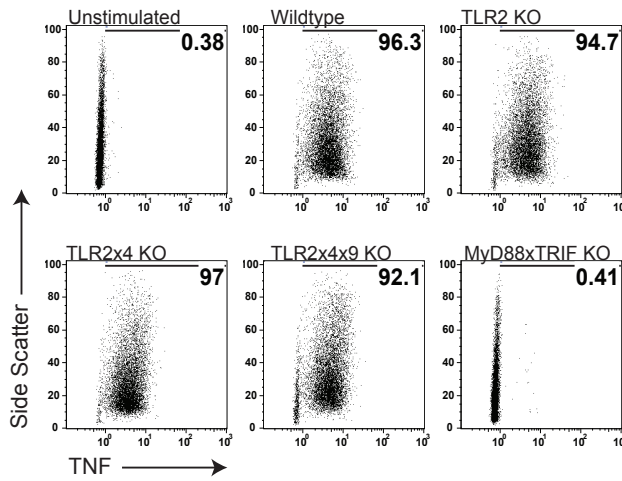


**Figure 2.1. Multiple TLRs recognize products of *S. typhimurium*.**

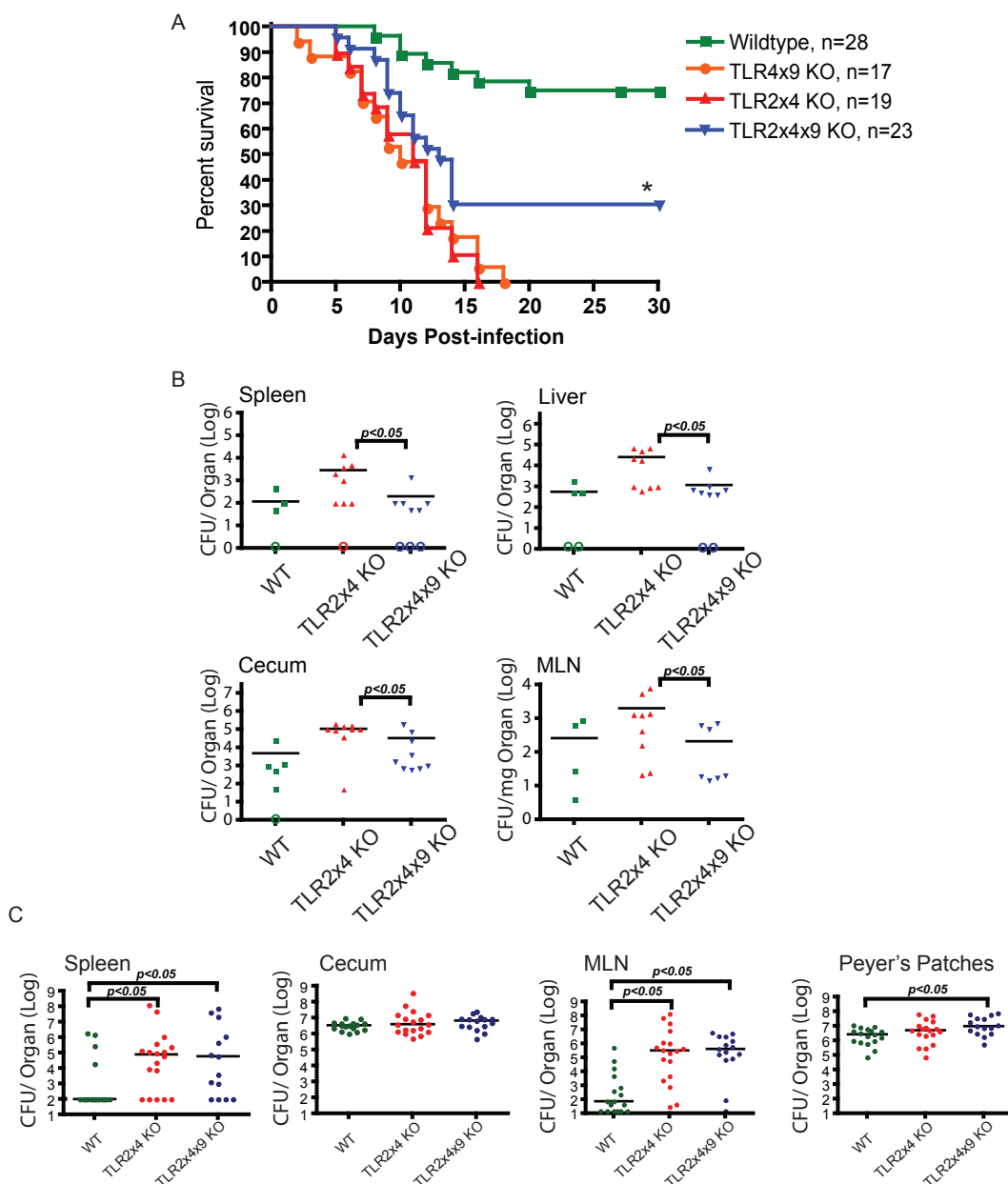
(A) HEK293 cells expressing the indicated TLRs or TLR accessory proteins together with an NF- $\kappa$ B luciferase reporter were treated with heat-killed wildtype LT2 *S. typhimurium* (*S.t.*), flagellin-deficient LT2 (*S.t.ΔfljB/fliC*), or genomic DNA isolated from flagellin-deficient LT2. Luciferase activity was measured after 8 hours. Relative MOI range: 100 to 3.125, DNA concentration: 375 ng/mL to 12.5 ng/mL. Data are representative of two independent experiments and shown as mean  $\pm$  SEM. LPS, lipopolysaccharide; Fla, flagellin; BLP, bacterial lipopeptide. (B) BMMs produce

reactive nitrogen in response to *S. typhimurium* via activation of multiple TLRs. BMMs differentiated from the indicated mice were treated overnight with 100 U/mL recombinant IFN- $\gamma$  and infected the next morning with complement-coated wild-type *S. typhimurium* (SL1344) at the indicated MOI. Nitrite production was measured 36h post-infection by Griess assay. Data are representative of 3 independent experiments and presented as mean  $\pm$  SD. **(C)** BMMs were infected as in (A) at an MOI of 5 for 8 hours, followed by intracellular cytokine staining for TNF. Percent TNF-positive cells are indicated in each panel. Data are representative of 3 independent experiments. **(D)** TLR7 and/or TLR3 are responsible for the remaining signaling in TLR2x4-KO BMMs. BMMs pre-treated for 2h with bafilomycinA1 or DMSO vehicle were infected with *S. typhimurium* (SL1344) at an MOI of 5 for 6 hours. Cells were processed and stained for intracellular cytokine staining as in (C). The percentage of cells positive for TNF staining is shown. See also Figure 2.2.



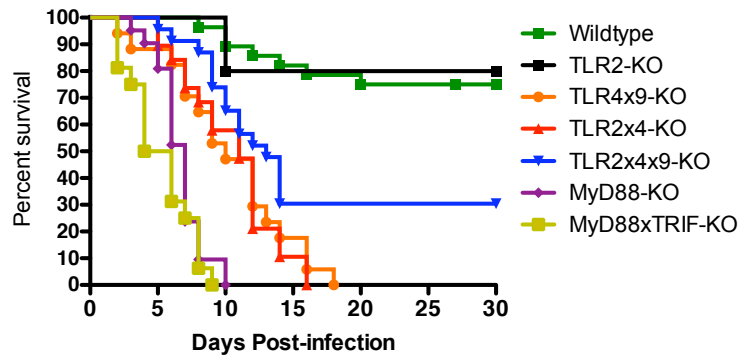


**Figure 2.2. BMMs of all genotypes respond equally to a TLR7 agonist.** BMMs from the indicated genotypes were stimulated with 100 ng/mL R848 for 8 hours, followed by intracellular cytokine staining for TNF. Percent TNF-positive cells are indicated in each panel. Data are representative of 3 independent experiments.

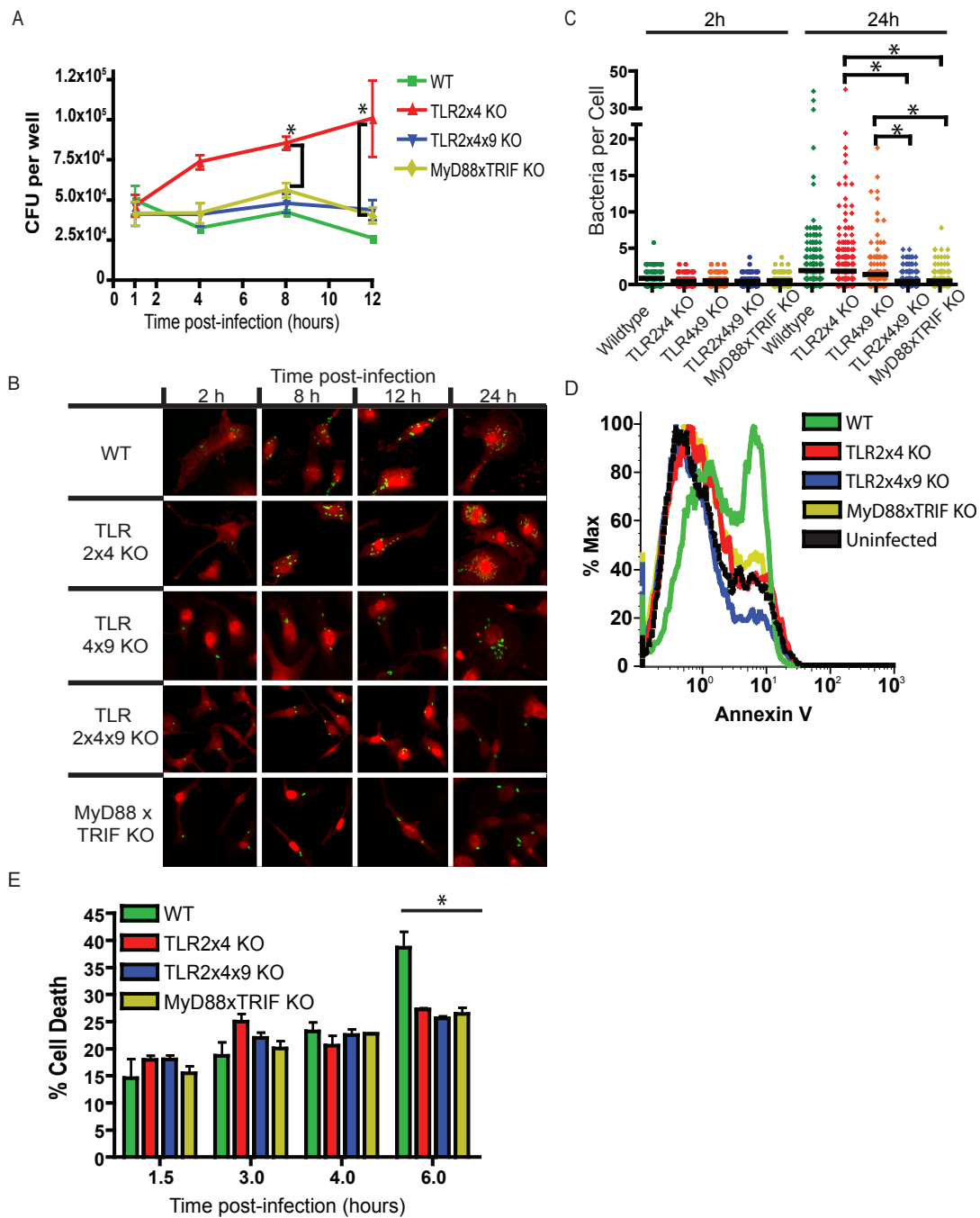


**Figure 2.3. TLR2x4x9-KO mice are less susceptible to *S. typhimurium* than TLR2x4-KO mice.** (A) A reduction in TLR signaling increases survival to *S. typhimurium* infection. Survival plots of mice orally inoculated with  $1.6 \times 10^8$  CFU *S. typhimurium* (SL1344) are shown. \*  $p < 0.005$  by log-rank curve comparison test. (B) TLR2x4x9-KO mice have reduced bacterial burden during *S. typhimurium* infection. Groups of 8-10 week-old mice were orally inoculated with  $1 \times 10^9$  CFU *S. typhimurium* (SL1344). Four days post-infection organs were harvested and homogenized for colony enumeration.

Data are representative of at least 3 independent experiments. (C) TLR2x4-KO and TLR2x4x9-KO mice are susceptible to *Y. enterocolitica* infection. Groups of mice of the indicated genotype were orally inoculated with  $2 \times 10^8$  *Y. enterocolitica* and CFU were measured in the indicated organs 3 days post-infection. For (B) and (C) bars represent mean CFU of all mice, with data significance determined by Mann-Whitney U test. Open circles indicate mice for which no colonies were detected. MLN, mesenteric lymph node. See also Figure 2.4.

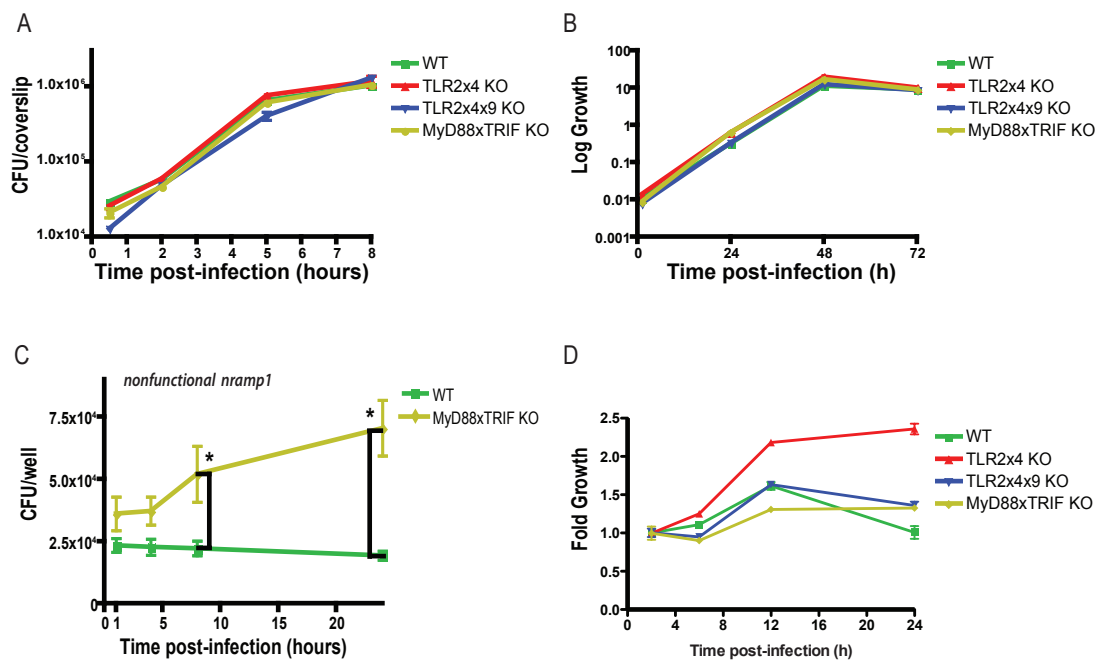


**Figure 2.4 Relative susceptibilities of TLR- or TLR adaptor-deficient mice to *S. typhimurium* infection.** Survival plots of mice orally inoculated with  $1.6 \times 10^8$  CFU *S. typhimurium* (SL1344) are shown.



**Figure 2.5. *S. typhimurium* is unable to replicate in TLR2x4x9-KO and MyD88xTRIF-KO BMMs.** (A) TLR signaling is required for intracellular replication of *S. typhimurium* in BMMs. BMMs derived from mice of the indicated genotypes were infected with *S. typhimurium* (SL1344) at an MOI of 1, and intracellular CFU was assessed via gentamicin protection intracellular replication assay at the indicated timepoints. Data are presented as the average of three independent experiments +/- SEM.

\*  $p < 0.05$  by student  $t$  test comparing TLR2x4-KO to MyD88xTRIF-KO and TLR2x4x9-KO. **(B)** BMMs deficient in TLR signaling have reduced numbers of bacteria per cell. BMMs infected as described in (A) were fixed and permeablized at the indicated times post-infection followed by staining with anti-*Salmonella* LPS antibody (green) and wheat germ agglutinin (red). **(C)** Intracellular bacteria per cell were counted in random fields at the 2 and 24 hour time-point from z-stacked images as shown in (B). Data are representative of two independent experiments,  $p$  value determined by student  $t$  test (\*  $p < 0.05$ ). **(D)** BMMs of the indicated genotypes were infected with *S. typhimurium* (SL1344) at an MOI of 5. 8h post-infection cells were harvested and stained with Annexin V. Data are representative of 2 independent experiments. **(E)** BMMs with intact TLR4 signaling undergo late cell death after infection with *S. typhimurium*. BMMs were infected at an MOI of 10 with *S. typhimurium* (SL1344), and release of lactate dehydrogenase (LDH) was measured in supernatants at the indicated time-points. Data are presented as mean  $\pm$  SD and are representative of at least 2 independent experiments  $p$  value determined by student  $t$  test (\*  $p < 0.05$ ) comparing wildtype to all other genotypes. See also Figure 2.6.



**Figure 2.6. Other intracellular pathogens can replicate in TLR2x4x9-KO and MyD88xTrif-KO BMMs.** (A) *Listeria monocytogenes* replicates in the absence of TLR signaling. BMMs from the indicated genotypes were infected at an MOI of 0.5 and monitored for intracellular replication at timepoints post-infection by gentamicin intracellular replication assay. (B) BMMs deficient for TLR signaling support growth of *Legionella pneumophila*. BMMs from the indicated genotypes were infected at an MOI of 0.01 with a  $\Delta$ *fla* *Legionella pneumophila* strain expressing the lux operon. Intracellular growth was monitored by increase in luminescence at varying timepoints post-infection. (C) Reliance on TLR signaling for intracellular growth requires functional Nramp1. MyD88xTRIF-KO BMMs with a non-functional copy of the *Nramp1* allele were infected with complement coated *S. typhimurium* (SL1344) at an MOI of 1. Intracellular growth at the indicated timepoints post-infection was monitored by gentamicin intracellular replication assay. (D) Intracellular growth of *S. typhimurium* (12023) in BMMs of the indicated genotypes.

## Discussion

Biological interactions are strong drivers of evolution, and the dynamics of host-pathogen interactions provide some of the clearest examples of this principle. Hosts have evolved resistance mechanisms, such as TLRs, that work by reducing pathogen fitness and drive the evolution of pathogen virulence (Hedrick, 2004; Rausher, 2001; Woolhouse et al., 2002). While virulence genes provide a fitness advantage, they can be energetically costly and often serve as targets of host sensors (Miao et al., 2010; Vance et al., 2009). Therefore, the ability to regulate expression of virulence genes based on changing environments is a key feature of microbial pathogenesis. In this study, we report the requirement for TLR signaling by the intracellular pathogen *S. typhimurium* to establish a successful infection and cause disease. We demonstrate that this requirement stems, at least in part, from the need for TLR-dependent phagosome acidification to induce SPI-2 genes, resulting in replication and virulence of the microbe. In the absence of sufficient TLR signaling, *S. typhimurium* is unable to create an SCV in BMMs and is less virulent *in vivo*. These data demonstrate that a pathogen can evolve to require innate immune signaling for full virulence. There is certainly experimental evidence demonstrating that host genetic variation can result in prolonged survival upon infection; however, these phenotypes are generally attributable to reduced inflammation and immunopathology, despite increased replication of the pathogen, suggesting that a reduction in resistance results in an elevation in tolerance (Gowen et al., 2006; Raberg et al., 2007). By contrast, our work demonstrates that mice lacking sufficient TLR signaling are less susceptible to a *S. typhimurium* infection due to reduced bacterial growth. A similar observation has been reported in *Drosophila*, where mutants in the melanization arm of the innate immune response are less susceptible to a *Streptococcus pneumoniae* infection compared to wildtype flies and this is associated with reduced levels of bacteria (Ayres and Schneider, 2008). However, it remains to be determined if *S. pneumoniae* requires the melanization response for growth or if melanization deficient flies have a heightened resistance response against the bacteria. Our work suggests that *S. typhimurium* has evolved to require innate immune signals to effectively coordinate expression of virulence genes required for intracellular replication and pathogenesis.

### *Cross-talk between Nramp-1 and TLR signaling*

Two aspects of our approach were crucial for our ability to observe the requirement for TLR-dependent signals in *Salmonella* virulence. First, by using mice that are deficient in multiple TLRs, as opposed to mice lacking MyD88 and TRIF, we were able to circumvent the susceptibility associated with the lack of IL-1 receptor family function. Indeed, the difference in susceptibility between MyD88xTRIF-KO and TLR2x4x9-KO mice that we observe underscores the importance of the IL-1R family in defense against infection and agrees with recent findings by several groups (Mayer-Barber et al., 2010; Raupach et al., 2006). Lack of TLR2, TLR4, and TLR9 eliminated most TLR-based recognition of *S. typhimurium* in macrophages, although a small amount of residual signaling was mediated by TLR7 and/or TLR3. We were somewhat surprised by the role for nucleic acid sensing TLRs in innate recognition of *Salmonella*. The role for these receptors suggests that even in cells in which *S. typhimurium* is replicating, bacterial nucleic acid is released. While the simplest explanation for this observation is that a small number of bacteria are degraded, it is also possible that a nucleic acid ligand



is secreted by *Salmonella* or present on the bacterial surface (Whitchurch et al., 2002; Woodward et al., 2010).

A second critical aspect of our study is that we used mice with a functional allele of *nramp1*. Only in the presence of functional Nramp1 is the dependence on TLR signaling for virulence evident. For example, *S. typhimurium* can efficiently replicate in MyD88xTRIF-KO BMMs lacking Nramp1 (Figure 2.6). Nramp1 functions as a divalent metal transporter and pumps  $Mn^{2+}$  (and likely other cations) out of the phagosome. Why the lack of this protein renders mice so susceptible to intracellular pathogens remains unclear, but this heightened sensitivity may simply obviate any requirement for TLR-dependent SPI-2 induction. The presence of functional Nramp1 has been shown to enhance SPI-2 expression as well as TLR-dependent responses (Fritsche et al., 2003; Valdez et al., 2008; Zaharik et al., 2002). Regardless of the precise mechanism responsible for the strong TLR-dependence when Nramp1 is functional, it is important to recognize that infection of cells with functional Nramp1 represents the “wildtype” scenario. Indeed, mutations in the human Nramp1 gene are associated with increased susceptibility to several intracellular pathogens (Bellamy et al., 1998; Malik et al., 2005). Therefore, examining *Salmonella* virulence in the presence of functional Nramp1 most accurately reflects the host-pathogen interactions between *S. typhimurium* and the mammalian immune system.

### **Chapter 3: TLR signaling enhances the rate of phagosomal acidification, a requirement for *Salmonella typhimurium* virulence gene induction.**

*Portions of this chapter were previously published (Arpaia et al., 2011).*

#### **Background**

As an intracellular pathogen *Salmonella* must survive within multiple cell types during an infection. *Salmonella* interacts with M cells overlaying Peyer's patches (Jones et al., 1994) and can actively induce its own entry into intestinal epithelial cells (Carter and Collins, 1974; Hohmann et al., 1978). After crossing the epithelial barrier, *Salmonella* is phagocytosed by macrophages, monocytes, neutrophils, and dendritic cells (DCs), and these cells may facilitate bacterial trafficking to systemic sites of infection (Clark et al., 1996; Everest et al., 1999; Vazquez-Torres and Fang, 2000; Vazquez-Torres et al., 1999).

After *Salmonella* crosses the intestinal lumen, virulence mechanisms enable the bacterium to survive and replicate within host phagocytes. Survival requires a distinct set of genes, many of which are encoded within *Salmonella* pathogenicity island 2 (SPI-2) (Galan, 2001; Shea et al., 1996; Waterman and Holden, 2003). SPI-2 encodes a type III secretion system (T3SS) whose expression is induced after the bacterium is phagocytosed by the host cell (Cirillo et al., 1998; Pfeifer et al., 1999; Valdivia and Falkow, 1997). Translocation of SPI-2 effectors into the host cell transforms the phagosome into a compartment that supports bacterial replication, the *Salmonella* containing vacuole (SCV) (Marcus et al., 2000). SPI-2 mutants fail to replicate in macrophages and cannot cause systemic infection, yet identification of the key host cells in which the bacterium utilizes SPI-2 to survive and replicate *in vivo* has been difficult. Multiple signals have been implicated in the transcriptional induction of SPI-2, including cation deprivation, phosphate starvation, and low pH (Chakravorty et al., 2005; Cirillo et al., 1998; Deiwick et al., 1999; Kim and Falkow, 2004; Rappl et al., 2003). However, most of the studies implicating these signals have been performed on bacteria grown *in vitro*; whether the same signals are responsible for induction of SPI-2 genes within the phagosome has been controversial.

Our studies presented in Chapter 2 indicated that intracellular growth of *Salmonella* is impaired in TLR2x4x9-KO and MyD88xTRIF-KO BMMs. This suggested that the defect may be related to poor SCV formation, and hence, an inability to activate SPI-2. However, the causal relationship between these two observations remained unclear, as a number of mechanisms could account for either phenotype. Therefore, in the following Chapter, we sought to define the underlying basis for impaired growth in BMMs lacking TLR function by visualizing SCVs by electron microscopy and performing global gene expression profiling of bacteria isolated from BMMs of each genotype.

Electron microscopy showed that in the absence of TLR signaling SCV formation was defective. For gene expression analysis, we performed quantitative RT-PCR to measure expression of all genes (approximately 4800 ORFs) in the *Salmonella* genome. Using K-means clustering analysis, we identified subsets of genes with differential expression profiles between the TLR2x4-KO and TLR2x4x9-KO samples. A large number of genes within the SPI-2 locus were upregulated in bacteria in TLR2x4-KO BMMs but not in bacteria in TLR2x4x9-KO and MyD88xTRIF-KO BMMs. To identify the TLR-dependent signal required by *Salmonella* for SPI-2 induction, we used a strain

with an HA-tagged allele of the SPI-2 effector, PipB2, and measured induction and secretion of this protein in macrophage lysates. Using pharmacological inhibitors to block potential signals we measured the effect on PipB2 induction and secretion.

Based on these data, we hypothesized that the lack of SPI-2 induction in TLR2x4x9-KO and MyD88xTRIF-KO BMMs is due to failure of SCVs to acidify. The issue of whether TLR signaling influences the kinetics of phagosomal maturation remains controversial (Blander and Medzhitov, 2004; Blander and Medzhitov, 2006a; Blander and Medzhitov, 2006b; Russell and Yates, 2007; Yates and Russell, 2005). To investigate this issue, we used ratiometric imaging to measure the pH of *Salmonella* containing phagosomes in BMMs of each genotype.

In this Chapter, we make a connection between TLR signaling and rapid phagosomal acidification. Phagosomal acidification is detected by the bacterium and used as a signal to induce SPI-2 gene expression. Hence, in the absence of TLR signaling, SPI-2 genes are not expressed in a timely enough manner to avoid degradation of the bacterium in the phagocytic compartment (Arpaia et al., 2011).

## **Materials and methods**

### ***Bacterial Strains and Infections***

*S. typhimurium* cultures were inoculated from single colonies and grown shaking at 250 rpm overnight in LB (Fisher Scientific) supplemented with 200 µg/mL streptomycin (in the case of SL1344, Invitrogen) at 37°C. The following morning, a 1:10 dilution of the quantified culture (in PBS, Gibco) was shaken in 25% normal mouse serum (Jackson ImmunoResearch) for 25 minutes, washed twice in PBS, and added to antibiotic free RPMI-1640 culture medium (above) for infection at the indicated MOIs. Cells were spin-infected for 5 minutes at 750 rpm, then incubated at 37°C for 25 minutes. Following incubation, cells were washed twice with PBS before the addition of 10µg/mL gentamicin-containing media. For MOIs greater than 10, cells were first incubated in 100µg/mL gentamicin-containing media for 1h, followed by a reduction to 10µg/mL gentamicin for the remainder of the experiment. For intracellular CFU determination, cells were washed once with PBS at the indicated time post-infection and lysed in 1% Triton-X 100 in PBS for 5 minutes at 37°C. Lysates were diluted to 0.2% Triton-X 100 with antibiotic-free complete RPMI, and dilutions were plated on LB agar plates containing 200 µg/mL streptomycin for colony enumeration. *PipB2-2xHA* (12032) and wildtype 12032 were kind gifts from S. Meresse. LT2 and SL1344, were obtained from S. Falkow (Stanford University).  $\Delta$ *fliB/fliC* (LT2) was a gift from R. Vance (UC Berkeley). *L. monocytogenes* was a gift from D. Portnoy (UC Berkeley). Constitutive SPI-2,  $\Delta$ *hha* (SL1344), was constructed by recombination using the  $\lambda$  Red recombinase method (Datsenko and Wanner, 2000) and phage transduction. For *L. monocytogenes* infections, BMMs were plated onto coverslips overnight and infected the following morning at an MOI of 1. Intracellular growth was monitored by gentamicin intracellular replication assay, as described (Leber et al., 2008). For *L. pneumophila* infections, BMMs were infected at an MOI of 0.05 with a *L. pneumophila* ( $\Delta$ *fla*) strain expressing the *lux* operon. Growth was monitored by increase in luminescence over time, as described (Lightfield et al., 2008).

### ***Electron Microscopy***

BMMs were plated on poly-D lysine-coated aclar slips. The following day, cells were infected at an MOI of 10 with wildtype or *ssaV::Kan* SL1344 (as described above, with addition of gentamicin). At the indicated timepoints post-infection, cells were fixed with 2% glutaraldehyde (EMS) in 0.1M phosphate buffer, pH 7.2 for 2 hours. Slips were washed 3 times with phosphate buffer followed by incubation for 30 minutes in 1% OsO<sub>4</sub> at room temperature in the dark. Following OsO<sub>4</sub> treatment, slips were washed 3 times with water then stained overnight with 0.5% sterile-filtered aqueous uranyl acetate at 4°C in the dark. The following day, washed slips were dehydrated using a progressive lowering of temperature ethanol dehydration, with final incubation in acetone. Cells were infiltrated and embedded in a graded series of epon-araldite resin, and sectioned using a Reichert Ultracut E microtome. Stained sections were imaged using an FEI Tecnai 12 transmission electron microscope with an accelerating voltage of 120 kV.

### ***S. typhimurium Effector Secretion***

BMMs were seeded in 6-well plates at  $2 \times 10^6$  cells per well and infected at an MOI of 25 with *S. typhimurium pipB2-2xHA* (12032). At the indicated timepoints post-infection, cells were washed twice with ice-cold PBS and lysed in 1% NP-40 in PBS in

the presence of protease-inhibitor cocktail (Roche) and EDTA (Fischer) for 30 minutes on ice. Lysates were cleared by centrifugation for 20 minutes at 13,200 x g, and 15% of the lysate was taken for detection of host cell controls while the remaining pellets were resuspended in 1X SDS buffer to monitor bacterial levels within the cells. The remaining supernatants were immunoprecipitated overnight with rat anti-HA agarose beads (blocked in 1% BSA PBS, Roche), washed four times the following morning with 1% NP-40 PBS, and run on a discontinuous 10% SDS PAGE gel. Gels were transferred onto immobilon transfer membrane (Millipore), blocked in 5% milk and probed with rat anti-HA (Roche) and goat anti-rat HRP (GE Healthcare). Gels were also run for the whole cell lysate and centrifugation pellets and transferred and probed as above with mouse anti-tubulin (EMD-Calbiochem) and mouse anti-DnaK (Stressgen/Assay Designs), respectively. For experiments where inhibitors were used, cells were pretreated for 1 hour with either BafilomycinA1 (50 nM, EMD-Calbiochem), APDC (100  $\mu$ M, Sigma Aldrich), Apocynin (1 mM, EMD-Calbiochem), L-NIL (1 mM, Sigma Aldrich), 10  $\mu$ g/mL cycloheximide (CHX), or vehicle control, then infected in the presence of each corresponding inhibitor and incubated with gentamicin containing each inhibitor for the remainder of the experiment following washes with PBS.

#### ***Whole-genome quantitative PCR analyses***

BMMs were seeded at  $1.5 \times 10^7$  cells per 75 cm<sup>2</sup> flask and infected at an MOI of 5 in 7 mL non-antibiotic complete RPMI (as above, with gentamicin addition after infection). At 2, 4, and 6 hours post infection, media was removed and cells were lysed in Trizol RNA reagent (Invitrogen). RNA was purified using PureLink<sup>TM</sup> Micro-to-Midi total RNA purification system (Invitrogen) as per manufacturer's instruction for isolation from Trizol.

RNA samples were treated for residual DNA contamination using Ambion Turbo DNA-free DNase (Invitrogen) as per manufacturer's instructions. Purified RNA was quantitated on a Nanodrop 1000 (Thermo Scientific) and visually inspected on a 1% agarose gel. RNA was reverse transcribe to cDNA for qRT-PCR experiments by adding 10  $\mu$ g of total RNA in a mixture containing random hexamers (Invitrogen), 0.01 M dithiothreitol, 25 mM dNTP mixture (Sigma), reaction buffer and 200 units of SuperScript III reverse transcriptase (Invitrogen) at 42°C for 2 hours. The RNA template then was hydrolyzed by adding NaOH and EDTA to a final concentration of 0.2 and 0.1 M, respectively, and incubating at 70°C for 15 min. cDNA was purified with a Minelute column (Qiagen) as per manufacturer's protocol. cDNA was eluted into 100  $\mu$ l of dH<sub>2</sub>O, diluted 1:50 in dH<sub>2</sub>O and mixed with an equal volume of Roche 2x SYBR master mix (Roche). cDNA/mastermix samples were aliquoted into Roche 384-well plates containing lyophilized primer pairs using a Biomek FX<sup>P</sup> Laboratory Automation Workstation (Biomek). Plates were subjected to centrifugation at 1000 rpm for 1 minute and stored at 4°C in the dark until ready for use.

Primer pairs were designed to ensure no secondary structures, a length of roughly 20-nucleotides and a melting temperature of 60°C using the primer design software Primer 3. All primer sequences are listed in Supplemental Information. Forward and reverse primers were diluted to a working concentration of 0.125 $\mu$ M. Primer pairs were dispensed into Roche 384-well PCR plates using a Biomek FX<sup>P</sup> Laboratory Automation Workstation (Biomek) in duplicates or quadruplicates. Primer pairs were then

lyophilized in Roche 384-well PCR plates for downstream use. A total of 4,800 primer pairs designed to 4,825 ORFs were tested for the initial analyses. A subset of genes showing significant trends of differential expression between genotypes were validated by independent QPCR reactions performed on biological replicates. For example, based on the initial whole-genome analysis, we reanalyzed expression of all genes within the SPI-2 pathogenicity island as well as genes flanking the locus. 288 primer pairs designed to 288 ORFs (42 of which belong to SPI-2) were arrayed in quadruplicate wells for each sample.

Plates were assayed in LightCycler<sup>®</sup> 480 Real-Time PCR System using the 384-well format. Reaction conditions were as follow: one cycle at 95°C for 5min, fifty-five cycles of 95°C for 10 sec, 60°C for 15 sec and 72°C for 10 sec. Finally, analysis was followed by a PCR melt curve analysis.

For data normalization, quadruplicate repeat Ct values for each sample were averaged and normalized to Ct values of 134 genes that were present in all samples. For each genotype, the 2h sample served as a baseline, allowing for normalization of the 4h and 6h Ct values for each corresponding genotype. The final values were multiplied by a factor of -1 such that higher expression correlated with a positive value. Sample expression data were analyzed on MeV software (TIGR). K-means clustering was used on the initial whole-genome screen to discover trends that were later validated by subset analysis.

### ***Immunofluorescence and Fluorescent Video Microscopy***

Coverslips were coated with poly-d-lysine (Sigma), washed with water and allowed to dry for 1 hour. BMMs were plated onto coated slips and allowed to settle overnight in non-antibiotic media and infected the following day (as above) with *S. typhimurium* (SL1344) at an MOI of 5, with addition of gentamicin. At the indicated timepoints post-infection, coverslips were washed with PBS, fixed with 4% PFA in PBS and permeabilized with 0.5% Triton-X 100 in PBS. Fixed coverslips were then washed with 0.1% Triton-X 100 in PBS, and blocked in IF blocking solution (5% goat serum, 2% BSA, 0.1% sodium azide and 0.1% Triton-X 100 in PBS). Slides were stained in IF blocking solution with FITC-conjugated mouse anti-*Salmonella* antibody (clone 1E6, Santa Cruz Biotechnology) and Cy3-conjugated wheat germ agglutinin (Invitrogen). Stained cells were imaged on a Nikon E800 fluorescent microscope. For bacteria per cell enumeration, bacteria were counted in random z-stacked images at the indicated timepoints.

For pH determination and video microscopy, BMMs were plate on poly-d-lysine coated Lab-Tek II #1.5 coverglass 4-chamber slides (Nunc) overnight followed by infection with fluorescently-labeled *Salmonella* (SL1344). Bacteria were labeled by incubation with 1.5 mg/ml FITC (Sigma Aldrich) in 100 mM NaHCO<sub>3</sub>, pH 8, rotating for 20 minutes in the dark at room temperature. Bacteria were then washed twice with 100mM NaHCO<sub>3</sub>, and added at the appropriate concentration to phenol-free DMEM (supplemented as described above but with GlutaMAX instead of L-Glutamine) for spin-infection. Following infection, chambers were incubated at 37°C for an additional 5 minutes, washed extensively with PBS then incubated with phenol-free DMEM containing 10 µg/mL gentamicin for the remainder of the experiment. Chambers were

kept on ice prior to mounting on a Nikon TE2000 inverted fluorescent microscope with environmental control kept at 37°C and 5% CO<sub>2</sub>.

Three fields for each BMM genotype containing greater than 20 intracellular bacteria were imaged for 70 minutes. Images were acquired at 2 minute intervals with excitation at both 440nm and 490nm. Images were processed and individual intracellular bacteria were tracked over time using Imaris Scientific 3D/4D image processing and analysis software (Bitplane) with background subtraction. Fluorescence intensity values following excitation at 440nm and 490nm were used to determine the 490/440 ratios for each bacterium at each timepoint.

To determine absolute pH values, standard curves were generated for each field and for each genotype assayed during the experiment. Briefly, pH-buffered solutions containing 145 mM KCl, 10mM Glucose, 1mM MgCl<sub>2</sub>, 10 μM nigericin (added fresh) and 20 mM of either sodium acetate (pH4.0-5.0), MES (pH 5.5-6.5), or HEPES (pH 7.0-7.4) were added to each well and images were taken of each field, as above. To account for field-specific background a standard curve was generated for each field and genotype, and the data were fit to a fourth-order polynomial equation (Microsoft Excel). Using these standard curves, ratios determined during the experiment (normalized to a ratio corresponding to pH 7 for the first timepoint) were plotted against the polynomial function to determine absolute pH using MATLAB software (MathWorks). To generate the curves seen in Figure 3.4B, we used a bootstrap computation that repeatedly and randomly samples a large data set and calculates the average ratio at each time point and measured deviations for each of these values (MATLAB, MathWorks).

## Results

### ***TLR signaling is required for establishment of the SCV***

To better understand why *S. typhimurium* is unable to replicate in TLR2x4x9-KO and MyD88xTRIF-KO BMMs, we investigated the fate of bacteria in infected TLR2x4-KO, TLR2x4x9-KO, and MyD88xTRIF-KO BMMs by transmission electron microscopy (Figure 3.1). At 2h post-infection, bacteria were clearly visible in well-defined vacuoles in BMMs of all three genotypes (Figure 3.1A, black triangles). By 8h post-infection, bacteria in TLR2x4-KO BMMs remained largely unchanged, although evidence of replication was evident in certain instances (Figure 3.1B). In contrast, phagosomes containing bacteria in TLR2x4x9-KO and MyD88xTRIF-KO BMMs were quite distinct. The bacteria often appeared mottled or irregular in shape, and in many cases bacteria were surrounded by electron dense staining material consistent with lysosomal fusion (Figure 3.1, open triangles). In some instances, bacteria were no longer surrounded by membrane, suggesting they entered the cytosol (Figure 3.1, white triangles). Cytosolic bacteria have been previously associated with the inability of bacteria to form an SCV (Beuzon et al., 2000). By 22h post-infection, these differences were even more pronounced. Bacteria in TLR2x4-KO BMMs had clearly replicated and established a SCV, while bacteria in TLR2x4x9-KO and MyD88xTRIF-KO BMMs showed further evidence of phagolysosomal fusion and degradation (Figure 3.1C). In total, the images clearly demonstrate a defect in the ability of *S. typhimurium* to establish a replicative compartment in TLR2x4x9-KO and MyD88xTRIF-KO BMMs.

### ***Induction of SPI-2 gene expression requires TLR signaling***

Our studies thus far indicate that intracellular growth of *S. typhimurium* is impaired in TLR2x4x9-KO and MyD88xTRIF-KO BMMs and suggest that this defect may be related to inefficient SCV formation. However, the causal relationship between these two observations remained unclear, as a number of mechanisms could account for either phenotype. Therefore, we sought to define the underlying basis for impaired growth in BMMs lacking TLR function by performing global gene expression profiling of bacteria isolated from BMMs of each genotype. Such analyses have traditionally been performed using microarrays, but the low sensitivity of this approach typically requires an RNA amplification step that may distort the relative abundance of individual transcripts. To avoid this issue, we performed quantitative RT-PCR to measure expression of all genes (approximately 4800 ORFs) in the *S. typhimurium* genome (Figure 3.2A). Transcript levels were compared between total RNA samples isolated from TLR2x4-KO and TLR2x4x9-KO BMMs infected with *S. typhimurium* at 2 and 4 hours post-infection. See the Materials and Methods for a detailed description of this analysis.

Using K-means clustering analysis, we identified subsets of genes with differential expression profiles between the TLR2x4-KO and TLR2x4x9-KO samples. A large fraction of genes within the SPI-2 locus were upregulated in bacteria in wildtype and TLR2x4-KO BMMs but not in bacteria in TLR2x4x9-KO or MyD88xTRIF-KO BMMs. To validate these expression differences, we reanalyzed expression of each gene within the SPI-2 locus, as well as genes adjacent to the locus (as controls), using independent RNA samples from infected wildtype, TLR2x4-KO, TLR2x4x9-KO, and



MyD88xTRIF-KO BMMs. As shown in Figure 3.2B, 13 genes within the SPI-2 locus were upregulated in wildtype and TLR2x4-KO BMMs but not in TLR2x4x9-KO and MyD88xTRIF-KO BMMs. These 13 genes most likely underestimate the extent to which the entire SPI-2 locus is differentially expressed between BMM genotypes, as many genes were statistically excluded due to extremely low levels of message in TLR2x4x9-KO or MyD88xTRIF-KO samples. For most SPI-2 genes, induction was higher in wildtype BMMs relative to TLR2x4-KO BMMs (Figure 3.2C), suggesting that induction correlates with the strength of TLR signaling. Thus, the lack of intracellular replication in TLR-deficient cells may be due to a failure to upregulate SPI-2 genes.

These expression-profiling studies indicated that transcription of SPI-2 genes within BMMs depends on signals downstream of TLR activation. To view SPI-2 induction at the protein level, we utilized a strain of *S. typhimurium* (12023) with an HA-tagged allele of *pipB2*. 12023 displays the same dependence on TLR signaling for intracellular growth as SL1344 (Figure 2.6D). We monitored the induction and secretion of PipB2 in BMMs of each genotype by lysing infected cells and probing cleared lysates for the presence of PipB2. PipB2 was strongly induced and secreted in infected TLR2x4-KO BMMs (Figure 3.2D). In contrast, the levels of PipB2 were significantly reduced in TLR2x4x9-KO BMMs and barely detectable in MyD88xTRIF-KO BMMs, despite equivalent numbers of bacteria in all samples (indicated by DnaK levels). These data are consistent with our transcriptional analyses and indicate that TLR signaling is required for the induction and function of SPI-2 genes.

#### ***TLR-dependent induction of SPI-2 genes is required for intracellular growth***

Based on the data presented, we hypothesized that the impaired induction of SPI-2 genes in bacteria isolated from TLR2x4x9-KO and MyD88xTRIF-KO BMMs was responsible for the defect in SCV formation and intracellular replication in these cells. To test this hypothesis, we compared the fate of bacteria lacking a functional SPI-2 secretion system (*ssaV::Kan*) in BMMs of each genotype. As expected, SPI-2 mutant bacteria were unable to replicate in BMMs of any genotype (Figure 3.3A). Next, we visualized the fate of SPI-2 mutant bacteria in TLR2x4-KO BMMs by EM. At two hours post-infection, the SPI-2 mutant bacteria were in well-defined vacuoles, similar to wildtype bacteria (compare 2h panels in Figure 3.1A and Figure 3.3B). However, at 8 and 22 hours post-infection, the SPI-2 mutant bacteria had an irregular appearance and showed evidence of fusion with lysosomes. These features were quite similar to the appearance of wildtype bacteria in TLR2x4x9-KO and MyD88xTRIF-KO BMMs at 8 and 22 hours post-infection (see Figure 3.1B, C). Thus, ultrastructural analyses support the conclusion that the impaired SCV formation by wildtype bacteria in TLR2x4x9-KO and MyD88xTRIF-KO BMMs is due to lack of SPI-2 induction.

If the lack of intracellular growth in TLR2x4x9-KO and MyD88xTRIF-KO BMMs is due to failure to induce SPI-2 genes, then a *S. typhimurium* strain with constitutive expression of SPI-2 genes should regain the ability to grow in these cells. To test this possibility directly, we constructed a strain lacking *hha* ( $\Delta hha$ ), a negative regulator of SPI-2 genes (Silphaduang et al., 2007). Previous work has demonstrated that  $\Delta hha$  mutant strain expresses SPI-2 genes constitutively (Silphaduang et al., 2007). Therefore, we tested the ability of  $\Delta hha$  bacteria to replicate in BMMs of each genotype. Remarkably, the  $\Delta hha$  mutant bacteria replicated in TLR2x4x9-KO and MyD88xTRIF-

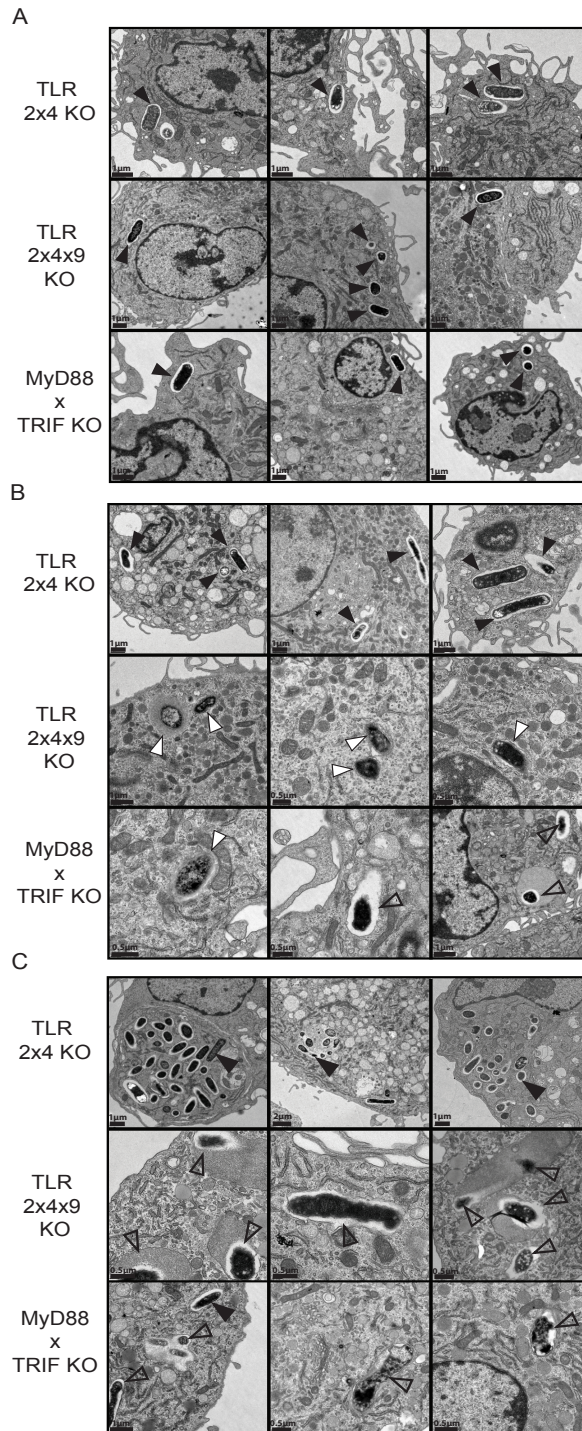
KO BMMs (Figure 3.3C). In fact,  $\Delta hha$  mutant bacteria replicated equivalently in BMMs of all genotypes (except wildtype cells where the lack of growth was due to TLR4-dependent cell death, as discussed earlier). While Hha most likely negatively regulates additional *Salmonella* virulence genes, the restoration of growth in TLR-deficient BMMs suggests that constitutive expression of SPI-2 genes completely restored intracellular bacterial replication. Thus, TLR signaling is required for upregulation of SPI-2 genes, and this requirement can be bypassed in a strain with constitutive SPI-2 expression.

### ***Induction of SPI-2 genes requires TLR-dependent acidification of the SCV***

Our results thus far indicate that TLR signaling provides a cue used by *S. typhimurium* to regulate SPI-2 expression. TLR activation leads to transcriptional induction of thousands of genes as well as more proximal effects, such as production of ROS and RNS and phagosome maturation and acidification, although this last aspect remains controversial. We used pharmacological inhibitors to block each of these potential signals and measured the effect on PipB2 induction and secretion. Treatment of TLR2x4-KO BMMs with cycloheximide (CHX) had no effect on PipB2 induction, indicating that host translation was not required for generation of the signal sensed by *S. typhimurium* (Figure 3.4A, bottom panel). Similarly, blocking ROS production with Apocynin or APDC or blocking RNS with L-NIL did not prevent PipB2 induction. However, inhibition of the V-ATPase with bafilomycinA1 blocked *S. typhimurium* induction of PipB2 in both TLR2x4-KO and wildtype BMMs. The block in TLR2x4-KO cells could be due to an inhibition of TLR signaling, as bafilomycinA1 almost completely inhibits the residual response to *S. typhimurium* (Figure 2.1D). In wildtype cells, though, TLR2 and TLR4 signaling is largely unaffected by bafilomycinA1 (Figure 2.1D), yet bafilomycinA1 treatment prevents PipB2 induction, suggesting that TLR-dependent acidification of the SCV may be the signal required by *S. typhimurium* for SPI-2 gene induction (Figure 3.4A, top panel). Experiments analyzing the induction of SPI-2 genes at the transcriptional level also indicated a requirement for phagosome acidification (data not shown).

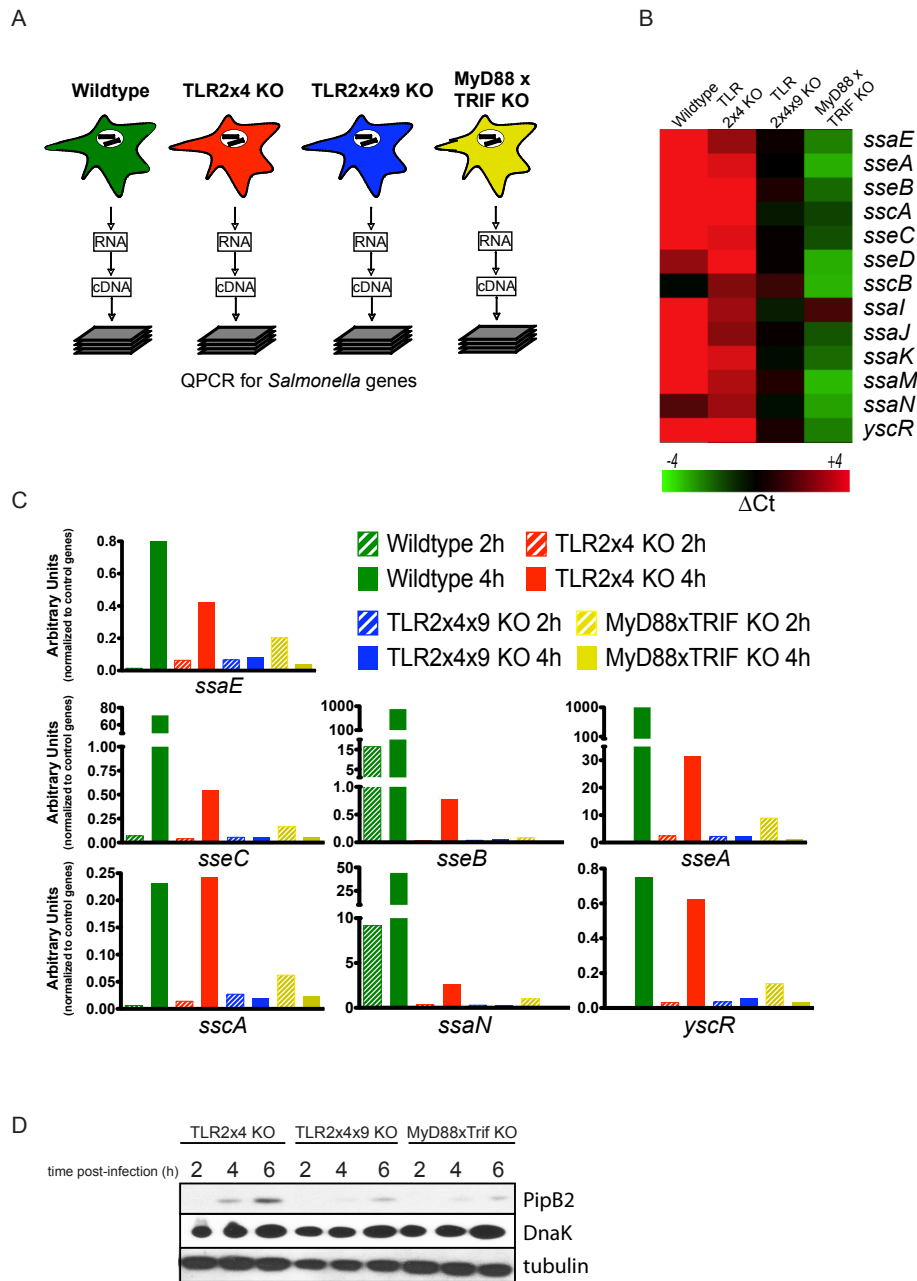
Based on these data, we hypothesized that the lack of SPI-2 induction in TLR2x4x9-KO and MyD88xTRIF-KO BMMs is due to failure of SCVs to acidify. While elegant studies have demonstrated that TLR-induced DC maturation is associated with recruitment of the V-ATPase to lysosomal membranes (Trombetta et al., 2003), the issue of whether TLR signaling influences the kinetics of phagosomal maturation remains controversial (Blander and Medzhitov, 2004; Blander and Medzhitov, 2006a; Blander and Medzhitov, 2006b; Russell and Yates, 2007; Yates and Russell, 2005). To investigate this issue in our system, we used ratiometric imaging to measure the pH of *Salmonella* containing phagosomes in BMMs of each genotype. This approach is based on the differential sensitivity to acidic pH of fluorescein excited at 490nm versus 440nm light and has been used to demonstrate that the SCV acidifies over time in wildtype macrophages (Rathman et al., 1996). We infected BMMs of each genotype with live *S. typhimurium* labeled with fluorescein, tracked the fluorescence of individual bacteria excited at each wavelength over time, and calculated the pH based on the  $I_{490}/I_{440}$  ratio (see Materials and Methods). While the mean pH of SCVs in wildtype and TLR2x4-KO BMMs dropped below 6 within 60 minutes post-infection, SCVs in TLR2x4x9-KO and

MyD88xTRIF-KO BMMs failed to acidify to the same extent and exhibited slower acidification kinetics (Figure 3.4B). By 30 minutes post-infection, over 70% of SCVs in wildtype and TLR2x4-KO BMMs had reached pH 6, while less than 35% of SCVs in TLR2x4x9-KO and MyD88xTRIF-KO BMMs had similarly acidified (Figure 3.4B). Consistent with the lower transcriptional induction of SPI-2 genes in TLR2x4-KO BMMs (relative to wildtype), the rate of acidification in TLR2x4-KO cells was slower than in wildtype cells, despite ultimately reaching pH 6 by 60 minutes. Collectively, these data support a model in which TLR signaling accelerates phagosomal acidification, which is used by *S. typhimurium* as a cue for SPI-2 gene induction.



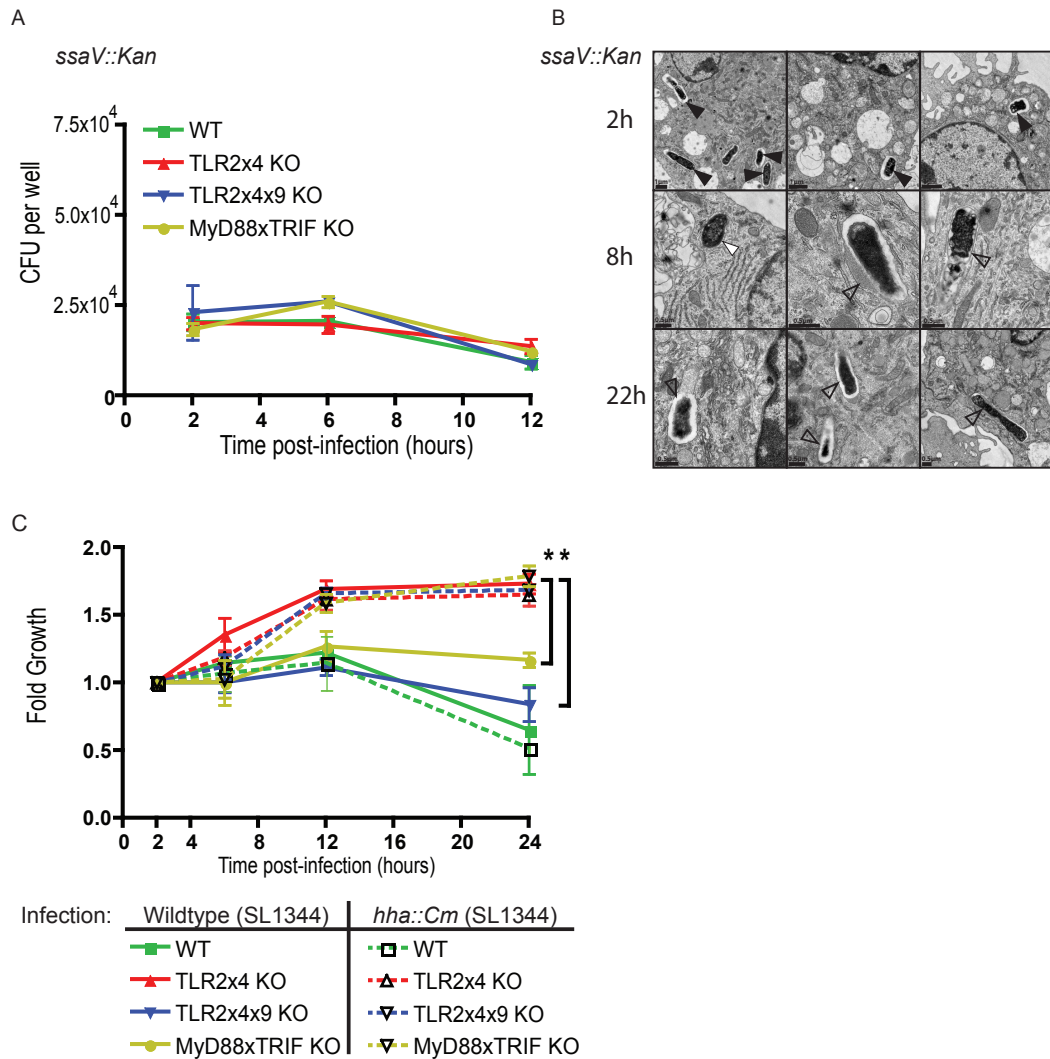
**Figure 3.1 *S. typhimurium* fails to form an SCV in TLR2x4x9-KO and MyD88xTRIF-KO BMMs.** TLR2x4-KO, TLR2x4x9-KO, or MyD88xTRIF-KO BMMs were infected at an MOI of 10 with *S. typhimurium* (SL1344), and cells were fixed and processed for electron microscopy at 2 (**A**), 8 (**B**), and 22 hours (**C**) post-infection. Bacteria in intact vacuoles are shown with filled black arrowheads, cytosolic bacteria

with filled white arrowheads, and bacteria that are degraded or have fused with lytic compartments are indicated with open black arrowheads. Micron bars are in the lower left corner of each panel. Three representative images from different sections and from independent infections are shown.



**Figure 3.2. *S. typhimurium* fails to upregulate and secrete SPI-2 effectors in TLR2x4x9-KO and MyD88xTRIF-KO BMMs.** (A) Schematic of quantitative expression analyses for all genes within the *S. typhimurium* genome. Total RNA was isolated from infected BMMs of the indicated genotypes followed by processing for quantitative RT-PCR (see Materials and Methods). (B) Heat map of normalized expression data for SPI-2 genes in bacteria within BMMs of the indicated genotypes (see schematic in (A)). For each BMM genotype, data are shown relative to the 2h timepoint. The gene designation is indicated to the right of each row. The data presented represent the mean of two independent experiments. (C) Relative induction of individual SPI-2 genes in bacteria isolated from BMMs of the indicated genotypes. Data are normalized

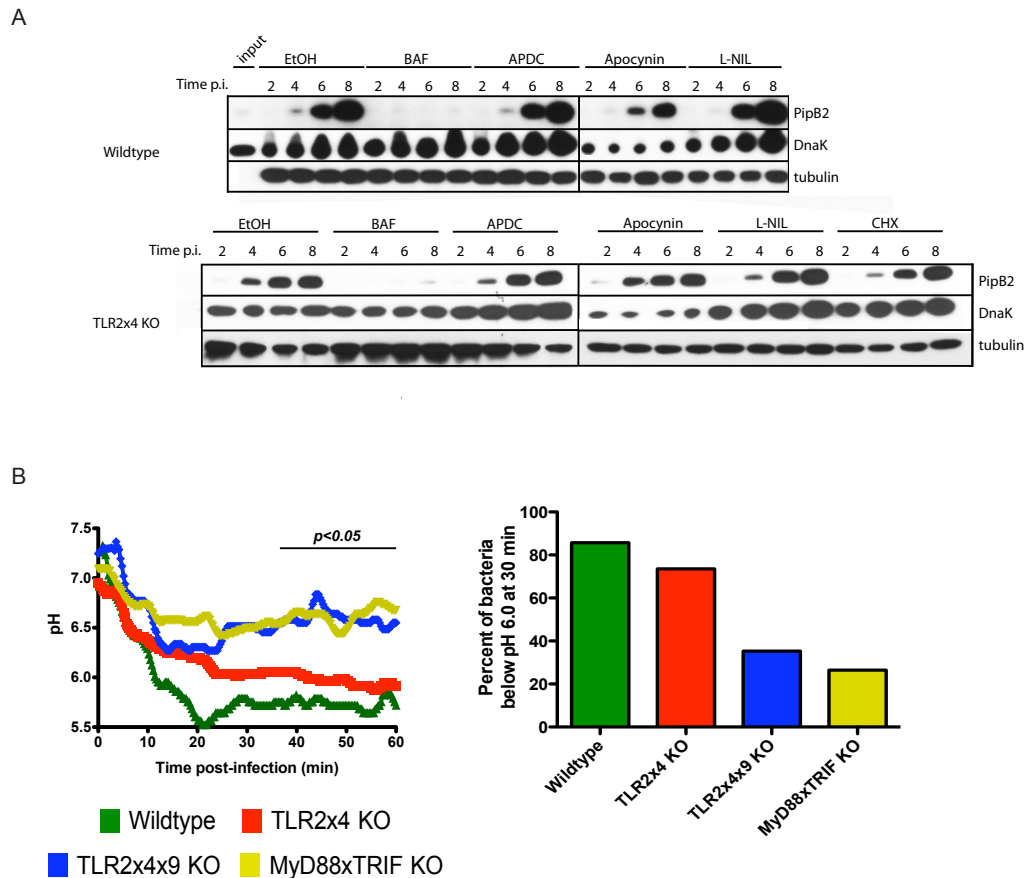
to the average expression values of a set of control genes. **(D)** TLR signaling is required for the induction and secretion of SPI-2 effectors. BMMs of the indicated genotypes were infected with an *S.typhimurium* strain (12032) expressing an HA-tagged allele of *pipB2* expressed from the endogenous *pipB2* locus. The presence of PipB2 in BMM lysates was detected by immunoprecipitation and immunoblot with anti-HA antibodies at 2, 4, and 6 hours post-infection. Controls for number of BMM (tubulin) and bacteria (DnaK) are also shown. Data are representative of 3 independent experiments.



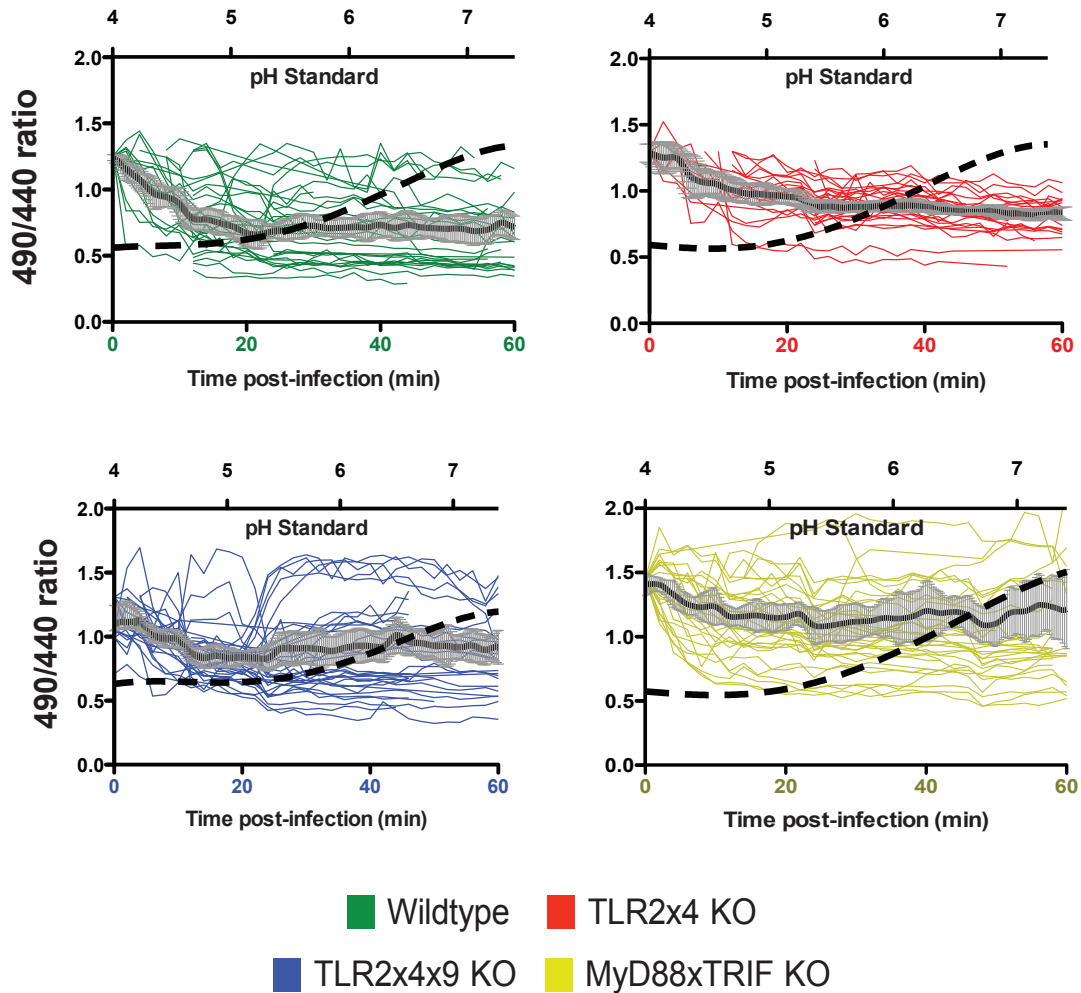
**Figure 3.3. TLR signaling is necessary for SPI-2 induction and intracellular growth.** (A) SPI-2 mutant bacteria fail to replicate in BMMs. BMMs of the indicated genotypes were infected with *ssaV::Kan* at an MOI of 1. Intracellular CFU were measured by gentamicin intracellular replication assay. (B) SPI-2 mutant bacteria fail to form an SCV in TLR2x4-KO BMMs. TLR2x4-KO BMMs were infected with a *ssaV::Kan* (SL1344) *S. typhimurium* strain followed by fixation and processing for electron microscopy at 2, 8 and 22 hours post-infection. Bacteria in intact vacuoles are shown with filled black triangles, cytosolic bacteria with filled white triangles, and bacteria that are degraded or have fused with lytic compartments are indicated with open black triangles. Three representative images from different sections and from independent infections are shown. (C) *Salmonella* mutants constitutively-expressing SPI-2 no longer require TLR signaling for intracellular growth and survival. BMMs of the indicated genotypes were infected with wildtype *S. typhimurium* (SL1344) or a strain with constitutive SPI-2 expression (SL1344 *hha::Cm*) at an MOI of 1. Intracellular CFU was measured via gentamicin



protection intracellular replication assay at the indicated timepoints post-infection. Data are presented as mean fold over the first time point (to control for minor inoculum differences between strains) +/- SEM and representative of 3 independent experiments. \*  $p < 0.05$  by student  $t$  test comparing TLR2x4-KO to MyD88xTRIF-KO and TLR2x4x9-KO at the indicated timepoint.



**Figure 3.4. TLR-dependent phagosomal acidification is required for SPI-2 expression.** (A) BMMs pre-treated with each of the indicated inhibitors, were infected with an *S.typhimurium* strain (12032) expressing an HA-tagged allele of *pipB2*. PipB2 levels in BMM lysates were detected by immunoprecipitation and immunoblot with anti-HA antibodies at 2, 4, 6 and 8 hours post-infection. Immunoblots for tubulin and DnaK serve as loading controls for BMMs and bacteria, respectively. Data are representative of 2 independent experiments for wildtype cells and 3 independent experiments for TLR2x4-KO BMMs. (B) TLR signaling accelerates phagosomal acidification. BMMs were infected with FITC-labeled *S.typhimurium* (SL1344) and the fluorescence intensities of individual bacteria excited at 490nm or 440nm were measured over time by live cell imaging. The fluorescence intensity ratio (490/440) reflects the pH within the phagosome (see Materials and Methods). The pH corresponding to each ratio was determined by generating a standard curve for each genotype of BMMs. The plots presented represent the mean pH calculated from at least 35 independent bacteria in multiple imaging fields. The right panel shows the percent of bacteria at or below pH 6 at 30 minutes post-infection. See Materials and Methods and Figure 3.5.



**Figure 3.5. Ratiometric imaging to calculate phagosomal pH.** Ratio measurements of individual bacteria within phagosomes in wildtype, TLR2x4-KO, TLR2x4x9-KO, or MyD88xTRIF-KO BMMs. Each thin line represents the 490/440 ratio of an individual bacterium over time. The solid black line represents the mean ratio as shown in Figure 3.4B with the 95% confidence interval indicated by gray bars. The dashed line represents the standard curve determined experimentally for each genotype (see Materials and Methods).

## Discussion

### *TLR signaling alters the pH of the Salmonella containing vacuole*

Our studies in BMMs derived from wildtype, TLR2x4-KO, TLR2x4x9-KO, and MyD88xTRIF-KO mice indicate that the difference in susceptibility of these mice is likely due to lack (or substantial delay) of SPI-2 induction. A number of signals have been proposed as inducers of SPI-2 expression, including low cation concentrations, phosphate starvation, and acidic pH (Chakravorty et al., 2005; Cirillo et al., 1998; Deiwick et al., 1999; Kim and Falkow, 2004; Rappl et al., 2003). Our results indicate that phagosome acidification is required for transcriptional induction of SPI-2 genes. Blocking acidification prevents SPI-2 induction, and measurements of phagosomal pH indicate that acidification is impaired and/or delayed in TLR-deficient cells. SPI-2 is also regulated post-translationally (Rappl et al., 2003; Yu et al., 2010). While we have not directly assessed whether TLR signaling impacts the post-translational stability of the SPI-2 apparatus, the fact that  $\Delta hha$  mutant bacteria can replicate in TLR2x4x9-KO and MyD88xTRIF-KO BMMs suggests that SPI-2 can function normally in the absence of TLR signaling.

Thus, we propose that TLR activation in wildtype and TLR2x4-KO cells results in acidification of the *Salmonella* containing phagosome. The extent to which TLR signaling influences phagosomal maturation (including increasing phagolysosomal fusion, acidification, and proteolytic activity) has remained a contentious issue (Blander and Medzhitov, 2004; Blander and Medzhitov, 2006a; Blander and Medzhitov, 2006b; Russell and Yates, 2007; Yates and Russell, 2005). While our studies were not designed to address this controversy, we clearly show that TLR signaling is required for rapid acidification of the SCV and has profound implications for the fate of intracellular bacteria and disease outcome. The mechanism leading to this induced acidification is likely similar to the basis of acidification of lysosomes during DC maturation, when TLR signaling leads to recruitment of the V1 subunit of the vacuolar ATPase to the lysosomal membrane (Trombetta et al., 2003). The precise signaling pathways downstream of TLR activation that lead to assembly of this machinery remain undefined. Moreover, whether bacteria sense pH directly or utilize other phagosomal features that require acidic pH for their generation remains unclear.

Importantly, we are not suggesting that phagosome maturation cannot occur without TLR signaling. Indeed, our EM images of infected TLR2x4x9-KO and MyD88xTRIF-KO BMMs at late time points (8 and 22h post-infection) show bacteria within electron dense compartments, suggestive of phagolysosomal fusion. Due to technical limitations we have not extended our pH measurements beyond 60 minutes post-infection, but our EM images suggest that phagosomes in TLR-deficient cells eventually mature. In fact, we do observe a small percentage of SCV in TLR2x4x9-KO and MyD88xTRIF-KO cells with significant reductions in pH within 1 hour (Figure S4). The lack of bacterial replication in TLR-deficient cells suggests that the eventual maturation of phagosomes is not sufficient to induce SPI-2 genes or that the induction occurs too late to prevent bacterial killing by lysosomal contents. It is also possible that the phagosome breaks down in the absence of SPI-2 function, and bacteria enter in the cytosol where they are unable to replicate (Beuzon et al., 2000). Our ultrastructural

analyses suggest that both of these possibilities may contribute to the lack of bacterial replication in TLR-deficient cells.

### ***Innate immune signaling as an environmental cue for virulence gene regulation***

The findings presented have important implications for our understanding of the evolution of host-pathogen interactions and virulence mechanisms. The antagonistic interactions between pathogens and host resistance strategies have driven the selection of an array of immunoevasive and immunosuppressive virulence mechanisms utilized by pathogenic microbes. Many pathogens trigger these mechanisms by utilizing signals downstream of innate receptors most likely as a reliable mechanism to ensure proper induction of genes necessary for survival in the presence of innate immune antimicrobial mechanisms. For example, the PhoP/PhoQ two-component system, when activated by antimicrobial peptides, induces expression of genes that modify lipidA and render the bacterial membrane more resistant to antimicrobial peptides (Guo et al., 1998). Our finding that *S. typhimurium* has evolved to require host resistance signals for proper expression of virulence genes is quite different than the currently known antagonistic strategies pathogens have evolved. Notably, *S. typhimurium* is unable to replicate in TLR-deficient cells, despite the absence of the antimicrobial mechanisms normally induced by TLRs. One implication of this remaining dependence is that the virulence genes induced by TLR signaling are required for purposes other than simply evading TLR-induced antimicrobial mechanisms to promote *S. typhimurium* fitness.

Why would *Salmonella* use signals downstream of TLRs to broadly coordinate expression of virulence genes required for intracellular growth? These signals may be the most reliable contextual cues that *Salmonella* can use to sense its presence within a macrophage phagosome. In general, a fundamental problem faced by *Salmonella* is the need to interact with multiple cell types through the course of an infection. Unique sets of virulence genes are required to survive each of these stages. As such, *Salmonella* is faced with the task of recognizing its environment and inducing relevant sets of virulence genes. For example, *Salmonella* must recognize when it has encountered a macrophage and induce SPI-2 genes, which are necessary for formation of the SCV and maintenance of the integrity of the phagosome. Precise regulation of such virulence genes is clearly essential for optimal growth, as mutant bacteria with constitutive expression of SPI-2 genes (e.g., *Δhha* mutants) are attenuated *in vivo* (Coombes et al., 2005; Silphaduang et al., 2007). Inappropriate expression of certain virulence genes could result in decreased fitness due to recognition by innate sensors or may disrupt proper regulation of other virulence genes required at specific stages of infection. Therefore, *Salmonella* utilizes TLR-dependent signals within the phagosome to detect its presence within a macrophage. Linking the induction of virulence genes (including SPI-2) to phagosomal signals downstream of TLRs may be an efficient way of coordinating multiple virulence mechanisms in response to a unifying contextual cue.

## Chapter 4: Other implications of reduced innate immunity in *Salmonella* infection

### Background

*Salmonella typhimurium* requires innate immune signaling to induce expression of its virulence genes encoded within SPI-2. We have previously shown that this induction is mediated by the rapid phagosomal pH reduction that occurs after TLR signaling (Arpaia et al., 2011). This model of reduced pathogenesis in the absence of TLR signaling was shown via oral administration of *Salmonella* and subsequent tracking of animal survival and bacterial dissemination to livers, spleens and mesenteric lymph nodes by CFU enumeration in these organs. SPI-2 is required for intracellular survival of *Salmonella* within phagocytic cells and it has previously been reported that any mutants in *Salmonella* that are unable to activate SPI-2 (and therefore are unable to replicate intracellularly) are extremely attenuated in vivo after both oral and intraperitoneal administration (Chakravorty et al., 2002; Cirillo et al., 1998; Hensel et al., 1997; Hensel et al., 1998). These analyses have always been done in animal backgrounds that are completely proficient in innate immune signaling, and therefore able to clear extracellular bacteria very rapidly.

The evolutionary requirement for *Salmonella* to replicate intracellularly must have arisen because of a fitness advantage gained by replicating within cells. Because expression of the T3SS requires a large input of energy for the bacterium, there must be some benefit of intracellular replication that cannot be otherwise bypassed. Neutrophils and inflammatory monocytes rapidly respond to invading pathogens, and much of this activation and recruitment is dependent upon activation of TLRs on neighboring cells and the infiltrating cells as well. In fact, studies have shown that neutralization of TNF- $\alpha$  after *Salmonella* infection can lead to extracellular replication of bacteria (Mastroeni et al., 1995). This suggested that selective pressures from the innate immune system may limit replication of *Salmonella* to intracellular compartments. Hence, SPI-2 induction is required for the bacterium to avoid detection and degradation by neutrophils and inflammatory monocytes that survey extracellular sites.

In the following Chapter, we sought to understand how pressures from the innate immune system limit *Salmonella* replication to intracellular compartments in vivo. Paradoxically, these same pressures that force intracellular replication as a means of evading the immune system are also required by the bacterium for induction of its virulence program that allows this intracellular replication. We therefore hypothesized that if we remove TLR signaling—thereby removing the ability for *Salmonella* to activate SPI-2 upon phagocytosis—that we could also remove the requirement for intracellular replication in vivo. More simply put, in the absence of TLR signaling, *Salmonella* can replicate extracellularly, and this replication should be independent of SPI-2.

Our initial analyses of survival after *Salmonella* infection showed that although there was a protection upon oral infection of TLR2x4x9-ko mice, this protection was not complete. We hypothesized that extracellular replication could explain the increased death in TLR2x4x9-ko mice compared to wildtype mice. To study this possibility, we took advantage of a membrane-impermeable antibiotic, gentamicin, to kill extracellular bacteria. Although preliminary, it seems that gentamicin delivery does in fact provide protection in TLR2x4x9-ko mice.

It was impossible to directly test extracellular replication in the absence of TLR signaling following oral inoculation because dissemination following this route of infection requires an intracellular replication intermediate. Because mice deficient for TLR signaling don't support intracellular replication (Arpaia et al., 2011), we needed to bypass this step to directly study extracellular replication in these animals. Therefore, we decided to repeat our experiments using intraperitoneal delivery of *Salmonella*. Further, we sought to generate mice in which there was even less TLR signaling than in TLR2x4x9-KO mice. To do this we generated mice lacking TLR2 and TLR4 but also harboring a single nucleotide polymorphism (3D mutation) that encodes a non-functional copy of Unc93b (a protein required for TLR3, TLR7, TLR8 and TLR9 signaling). Thus, we generated TLR2x4-KOxUnc93b<sup>3D/3D</sup> with a functional copy of *Nramp1*. Analysis of BMM from these mice showed that TLR signaling after *Samonella* infection phenocopies that in MyD88xTRIF-KO BMM. Extracellular replication in these animals was also tested.

## **Materials and Methods**

### ***Bacterial Strains and Infections***

*S. typhimurium* cultures were inoculated from single colonies and grown shaking at 250 rpm overnight in LB (Fisher Scientific) supplemented with 200 µg/mL streptomycin (in the case of SL1344, Invitrogen) at 37°C. *SsaV::Kan* SL1344 strain was provided by D. Monack (Stanford University) and prepared as above.

### ***Mice and in vivo Infections***

All animal experiments were carried out under approval from the animal care and use committee (ACUC) of the University of California. TLR2-, TLR4-, TLR9-, MyD88- and TRIF-deficient mice were generated and kindly provided by S. Akira (Osaka University). *Unc93b*<sup>3D/3D</sup> were purchased from UC Davis. Mice were intercrossed to generate strains lacking multiple genes. All strains were backcrossed onto the C57Bl/6 background while maintaining the functional *Nramp1* allele (G<sup>169</sup>). The degree of backcrossing was verified by SNP analysis comparing 129S1 to C57Bl/6 for all strains but TLR2x4x*Unc93b*<sup>3D/3D</sup>. Briefly, genomic DNA isolated from tails of backcrossed mice was purified for Illumina-based 129S1 vs. C57Bl/6 SNP detection performed by the Harvard-Partners Center for Genetics and Genomics (HPCGG). A total of 510 SNPs across the genome showed greater than 90% C57Bl/6 character as indicated by at least one allele of the C57Bl/6 SNP at each locus tested. The remaining 129S1 SNPs were within regions adjacent to loci of targeted genes (and therefore unlikely to be lost through backcrossing without a rare crossover event).

For survival and CFU enumeration experiments, age-matched mice were transferred to new cages and withheld food 14 hours before infection, unless infected intraperitoneally (as indicated). Animals were inoculated by gavage with 100 µL of the indicated CFU of *S. typhimurium* (SL1344), then given food and water *ad libitum*. For gentamicin administration, 4.5 µg gentamicin sulfate solution in PBS per gram of mouse was injected retro-orbitally starting on day 3 post-infection. Mice were injected with the same dose once per day after day 3 for the remainder of the experiment. For example, a 20 g mouse received 90 µL of 1 mg/mL gentamicin solution in PBS. For CFU enumeration, organs were harvested and homogenized in PBS using a Polytron PT2100 homogenizer at 17000 rpm (Kinematica), serially diluted, and plated on 200 µg/mL streptomycin (for *Salmonella*) or 1 µg/mL irgasan-containing (for *Yersinia*) LB-agar plates.

### ***Measurement of BMM activation***

For measurement of TNF production, BMMs were infected with bacteria or treated with TLR ligands (as described above). 30 min later, BMMs were treated with brefeldinA (eBiosciences). After an additional 3.5-4h, cells were harvested, fixed, permeabilized, stained with anti-TNF antibody (eBioscience), and analyzed on an FC500 flow cytometer (Beckman Coulter). Fixation and permeabilization was performed according to manufacturer's instructions (eBioscience). For infections, all steps prior to fixation were performed in the presence of 10µg/mL gentamicin.



## Results

### ***Intraperitoneal (IP) injection of *S. typhimurium* shows dependence on TLR signaling for bacterial clearance***

Our initial observation that TLR signaling is required for intracellular replication was seen after oral infection of mice. In order to determine whether intraperitoneal infection showed a similar dependence for TLR signaling, we infected mice intraperitoneally with 100 CFU of *S. typhimurium* (SL1344) and monitored CFU in spleens and livers 2 days post-infection. As can be seen in Figure 4.1A and B, mice showed an opposite phenotype than that observed after oral inoculation. Whereas wildtype (WT) mice are still able to clear *S. typhimurium*, any further reduction in TLR signaling makes mice more susceptible. This result was also apparent when comparing weight loss following infection (Figure 4.1C). In the oral inoculation experiments presented in Chapter 2, TLR2x4x9-KO mice were less susceptible to *S. typhimurium* infection.

To explain this observation we considered two possibilities. The first possibility is that upon intraperitoneal infection, the cell types encountered by *Salmonella* still have enough residual TLR signaling to activate SPI-2 genes and cause infection. The second possibility was that upon intraperitoneal infection in the absence of TLR signaling there is such reduced inflammation that *Salmonella* bypass the need for intracellular replication and can replicate and cause disease even without activating SPI-2.

To address the first possibility, we generated mice in which there was even further reduction of TLR signaling. To do this, we took advantage of a single nucleotide polymorphism in a gene call *Unc93b1*. This gene encodes a multipass transmembrane protein that is required for signaling of all intracellular TLRs (TLR3, TLR7, TLR8 and TLR9). When we crossed these mice onto the TLR2x4-KOxNrp1<sup>R/R</sup> background we generated mice in which the only remaining known functional TLR was TLR5, which is not expressed in BMM (Figure 4.2). We further tested to see if any residual TLR signaling remained in response to various bacteria by infecting these BMM and staining for intracellular TNF- $\alpha$ . As can be seen in Figure 4.3, In response to *E. Coli*, *S. typhimurium*, and *L. monocytogenes*, TLR2x4-KOxUnc93b<sup>3D/3D</sup> phenocopied MyD88xTRIF-KO cells, suggesting that we had eliminated signaling from all TLRs that are capable of recognizing these bacteria in BMM. To test whether or not these mice were protected from *Salmonella* infection delivered IP, we infected these mice as in Figure 4.1. Unfortunately, when we delivered 250 CFU of bacteria IP, all of the TLR2x4-KOxUnc93b<sup>3D/3D</sup> died by day 3 after infection, making us unable to perform CFU analyses.

### ***In the absence of TLR signaling *S. typhimurium* is able to replicate extracellularly and do not require SPI-2***

The fact that wildtype bacteria were highly virulent in TLR2x4-KOxUnc93b<sup>3D/3D</sup> did not formally distinguish between the two hypotheses we considered. There was still the possibility that bacteria were inducing SPI-2 genes independently of TLR signaling when delivered IP. In order to distinguish between bacteria replicating extracellularly or inducing SPI-2 genes independent of TLR signaling, we infected mice with a mixture of both SPI-2 knockout (*ssaV::kan*) and wildtype bacteria (each tagged by antibiotic resistance) and monitored relative replication of each strain after infection. If SPI-2

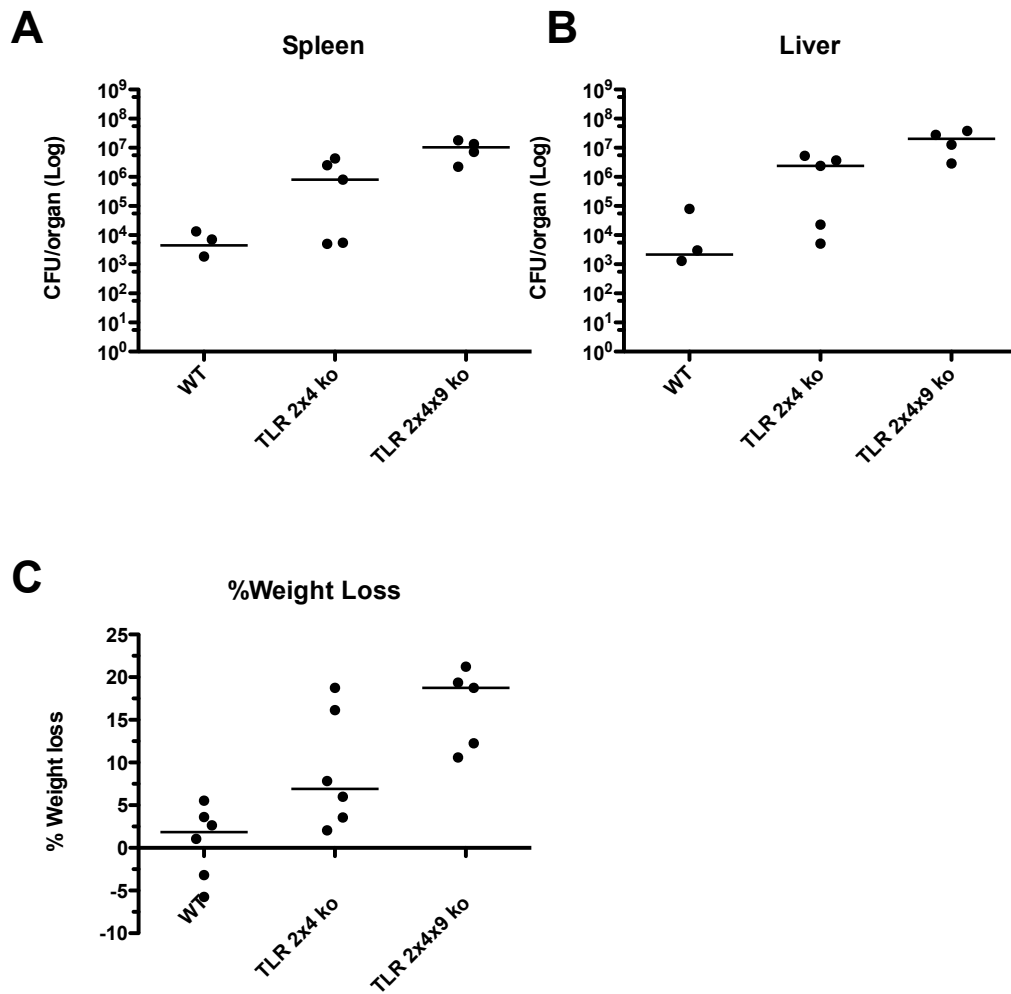
knockout bacteria were able to replicate after IP infection, this suggested that in the absence of TLR signaling (in TLR2x4-KOxUnc93b<sup>3D/3D</sup>) *Salmonella* was no longer constrained to an intracellular lifecycle and could replicate extracellularly. This result does not formally rule out that *Salmonella* is replicating within a cell type that does not require SPI-2, however (a point that will be considered later).

As expected for extracellular replication, in the absence of TLR signaling, SPI-2-deficient bacteria were still able to replicate to very high levels in spleens and livers of TLR2x4-KOxUnc93b<sup>3D/3D</sup> mice (Figure 4.4). In fact, all MyD88xTRIF-ko mice that received SPI-2 knockout bacteria alone still succumbed to infection and died within 3 days (data not shown). Whereas TLR2x4-KO and wildtype mice were able to almost completely clear SPI-2 knockout bacteria, TLR2x4-KOxUnc93b<sup>3D/3D</sup> exhibited 3-4 logs more of this knockout strain in spleens and livers (Figure 4.4). This result suggested that in the absence of TLR signaling *Salmonella* may be replicating extracellularly and could potentially account for why some TLR2x4x9-ko mice die following oral infection with wildtype *Salmonella*—if bacteria are able to access extracellular sites, then they rapidly replicate and cause lethality.

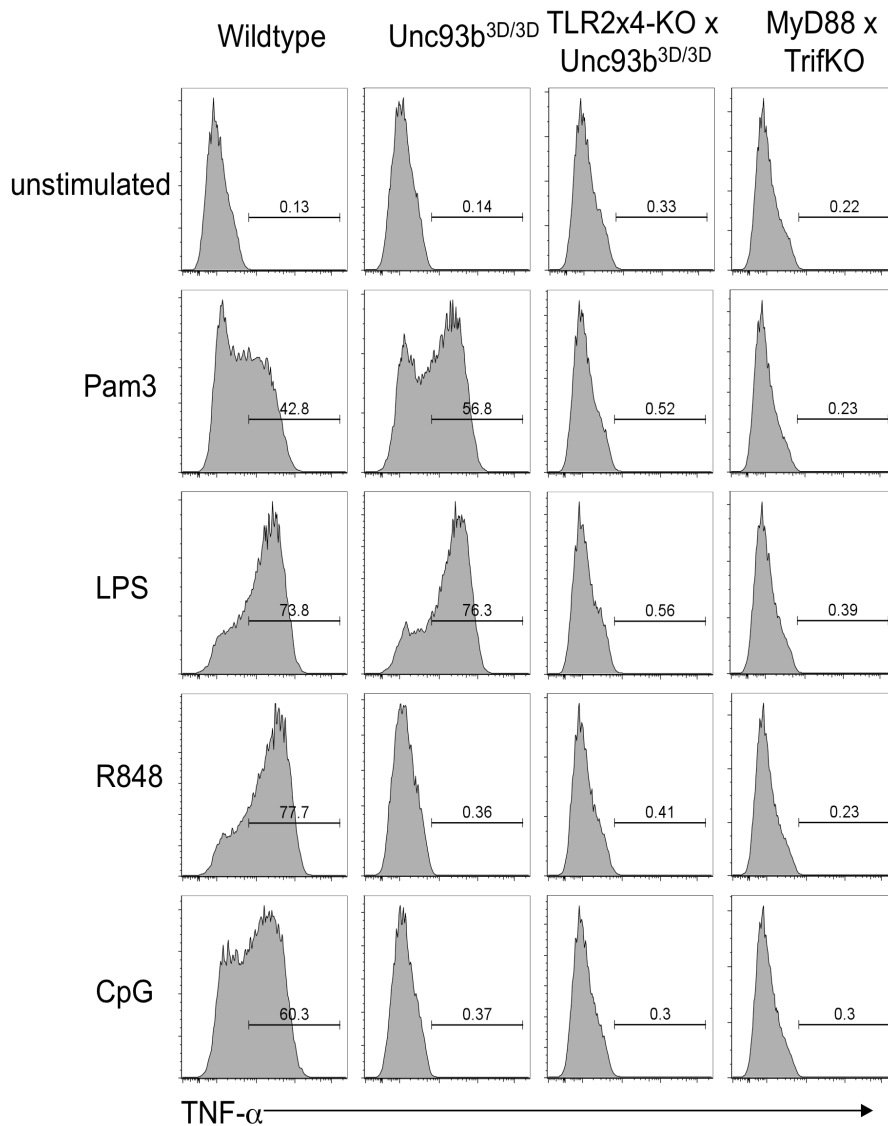
#### ***Clearance of extracellular bacteria by treatment with membrane-impermeable antibiotic rescues TLR2x4x9-KO mice***

There was still the possibility that, after IP infection, *Salmonella* lives within a cell type in which it doesn't require SPI-2 induction for survival. In order to formally show that extracellular replication accounts for death and increased CFU following infection of TLR-deficient mice, we took advantage of a membrane impermeable antibiotic, gentamicin, that specifically kills extracellular bacteria. As is shown in Figure 2.3, 70% of TLR2x4x9-KO mice still die after oral infection with wildtype bacteria. We hypothesized that this was due to some bacteria being able to access extracellular sites at late time points post-infection.

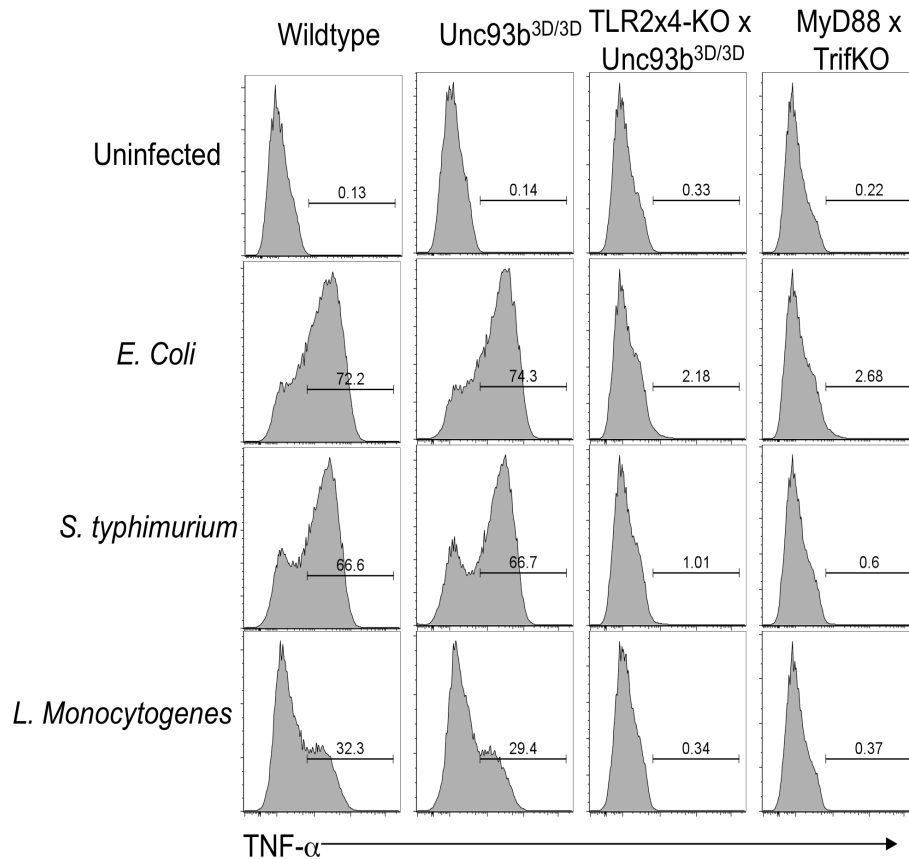
If we were able to selectively kill extracellular bacteria during this infection, then TLR2x4x9-KO mice should be protected because bacteria are not activating SPI-2 (and not replicating intracellularly) and are being killed upon exit to extracellular sites by gentamicin. In contrast, in TLR2x4-KO mice (which induce SPI-2 genes), mice should still succumb to infection because even though extracellular bacteria are being killed by gentamicin, replication is occurring intracellularly. There is still the possibility that we may see reduced lethality in TLR2x4-KO mice because a subset of these mice may also die because of extracellular replication. As is shown in Figure 4.5, with gentamicin treatment, all TLR2x4x9-KO mice are protected from lethality with oral *Salmonella* challenge. This is in contrast to TLR2x4-KO mice, where approximately 40% of the mice still die after infection. These data suggest that TLR signaling is required for two important steps during infection (one beneficial for the host and the other for the bacterium): 1) TLR signaling is required to induce expression of SPI-2 genes within *Salmonella* and allow intracellular survival and 2) TLR signaling is required to limit extracellular replication of bacteria and force an intracellular lifecycle.



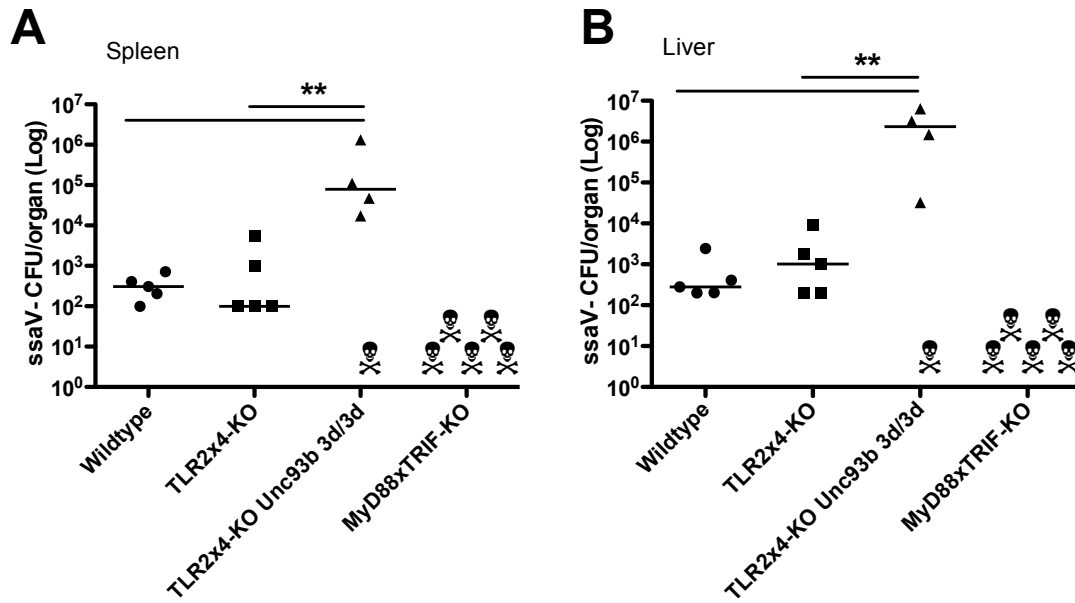
**Figure 4.1. TLR2x4x9-KO mice are more susceptible than TLR2x4-KO mice when infected by intraperitoneal injection.** Mice from the indicated genotypes were infected intraperitoneally with 100 CFU of *S. typhimurium* (SL1344) and organs were harvest 3 days post-infection. CFU retrieved from harvested spleens (A) and livers (B) are shown. (C) Percent weight-loss of animals comparing pre-infection to day 3 post-infection. Median are shown for all values.



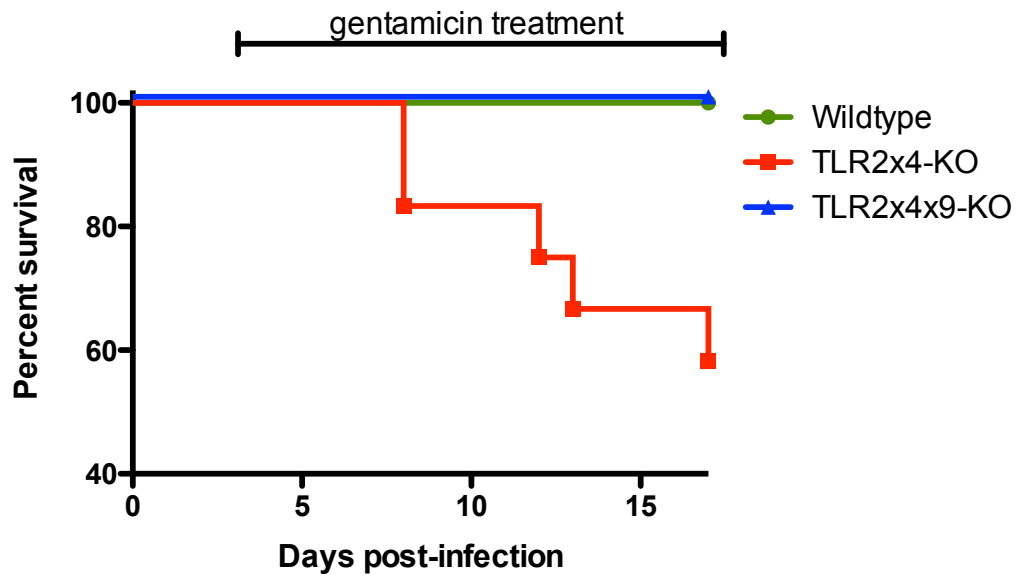
**Figure 4.2. BMM from TLR2x4-KOxUnc93b<sup>3D/3D</sup> mice behave like MyD88xTRIF-KO BMM upon stimulation with TLR ligands.** BMM from mice of the indicated genotypes were stimulated with each of the listed ligands and stained for intracellular production of TNF- $\alpha$  after 4.5 hours in Brefeldin A. Pam3 (TLR2), LPS (TLR4), R848 (TLR7) and CpG (TLR9).



**Figure 4.3. BMM from TLR2x4-KOxUnc93b<sup>3D/3D</sup> mice behave like MyD88xTRIF-KO BMM upon infection with different bacteria.** BMM from mice of the indicated genotypes were infected with each of the listed bacteria for 30 minutes, followed by addition of gentamicin to kill extracellular bacteria and Brefeldin A. Cells were then intracellularly stained for TNF- $\alpha$  after 4.5 hours in Brefeldin A.



**Figure 4.4. SPI-2 deficient bacteria are able to replicate and cause lethality in the absence TLR signaling.** Mice from the indicated genotypes were infected intraperitoneally with a mixture of 500 CFU of *S. typhimurium* (SL1344) and 500 CFU *SsaV::Kan* (SL1344) and organs were harvest 2 days post-infection. *SsaV::Kan* CFU (determined by selective growth on kanamycin plates) retrieved from harvested spleens (**A**) and livers (**B**) are shown. Skeletons represent mice that succumbed to challenge before harvest. \*\*p<0.05.



**Figure 4.5. TLR2x4x9-KO mice are protected from lethal *Salmonella* challenge by treatment with gentamicin to kill extracellular bacteria.** Mice from the indicated genotypes were orally inoculated with 1E9 CFU wildtype *S. typhimurium* (SL1344) after 14 hours of starvation. On day 3 post-infection mice were treated with 4.5  $\mu\text{g/g}$  mouse of gentamicin sulfate solution retro-orbitally. Mice were monitored for survival as described in Material and Methods.

## Discussion

The data presented in this Chapter highlight two very important aspects of TLR signaling for bacterial pathogenesis. As was already discussed in Chapters 2 and 3, TLR signaling is required for induction of SPI-2 genes within *Salmonella* by inducing a rapid phagosomal pH reduction upon bacterial engulfment. This reduction in pH is sensed by *Salmonella* and used to initiate a virulence program that has the overall outcome of permitting intracellular survival within phagocytic cells. Further, the data presented in this Chapter highlight another aspect of TLR signaling—namely activation and recruitment of cells to clear extracellular bacteria. The dichotomy between these two opposing activities, one allowing intracellular survival on the part of the bacterium, and the other forcing a bacterial intracellular lifecycle on the part of the host, most likely highlights the coevolutionary relationship between pathogen and host in response to selective pressures. As is stated by the red queen hypothesis, evolution of a host defense strategy is accompanied by the counter evolution of an evasion strategy by the pathogen.

Upon evolution of an innate immune system that served to kill and clear extracellular bacteria, *Salmonella* consequently needed to evolve a mechanism for evading this new arsenal of host defense. By committing to an intracellular lifecycle, and using the precise signal that drove this commitment to activate its intracellular survival program, *Salmonella* maximizes its fitness by only expressing virulence genes when it is completely necessary. The idea that host defense has driven the evolutionary requirement for acquisition of intracellular survival mechanisms—and, therefore systemic pathogenesis—has been loosely alluded to before (Baumler, 1997). What we have contributed to this idea is an experimental test of this hypothesis. Namely, when innate immunity is absent, the intracellular survival program is no longer needed and *Salmonella* can be pathogenic because it replicates undetected in extracellular compartments. Through the use of sophisticated mouse models that combine innate immune deficiency with membrane impermeable antibiotic treatment and bacterial genetics, we have provided evidence of this counter evolution strategy in vivo.

Much work still remains, however. The data presented here are still in their preliminary stages, and many questions are still unanswered. For example, TLR2x4-KOxUnc93b<sup>3D/3D</sup> are highly susceptible to *Salmonella* orally. We attribute this phenotype to rapid extracellular replication of bacteria, and in fact, treatment with gentamicin is capable of curing these mice of lethality. However, the concentration of gentamicin needed in order to counteract this rapid extracellular replication also partially inhibits pathogenesis in TLR2x4-KO mice. This high concentration most likely represents the half-life of gentamicin and its inability to inhibit the incredibly rapid replication of *Salmonella* in the complete absence of TLR signaling. Although the gentamicin protocol may not be a viable way of showing extracellular replication in TLR2x4-KOxUnc93b<sup>3D/3D</sup>, the replication of SPI-2 deficient *Salmonella* in these mice is certainly strong evidence that extracellular replication does indeed occur and is the cause of lethality.



## Chapter 5: Future Directions and Closing Remarks

### Future Directions

Many questions still remain regarding the relationship between TLR activation and *Salmonella* pathogenesis. For example, the profound contribution of Nramp1 to the dependence on TLR signaling for SPI-2 activation is difficult to understand, however, there are two potential hypotheses to explain this observation. 1) In the Nramp<sup>R/R</sup> background, functional Nramp could be removing a signal for SPI-2 activation that is otherwise provided by TLR signaling. This could explain why, even in the absence of TLR signaling, bacteria are able to replicate in MyD88xTRIF-KO BMMs on the Nramp<sup>S/S</sup> background. This signal could still be pH reduction, suggesting that in the Nramp<sup>S/S</sup> background phagosomal pH decreases independently of TLR signaling, or with fast enough kinetics to induce SPI-2 activation. 2) A second potential hypothesis is that in the absence of functional Nramp *Salmonella* no longer requires SPI-2 to replicate intracellularly. Both of these hypotheses can be easily tested in vitro by doing growth curves with wildtype and SPI-2 deficient bacteria in MyD88xTRIF-KO BMMs from both Nramp<sup>R/R</sup> and Nramp<sup>S/S</sup> backgrounds. Also, monitoring pH reduction in each of these BMMs following infection will allow us to determine if phagosomal acidification occurs with different kinetics (potentially faster) in the absence of functional Nramp.

In terms of phagosomal pH reduction, it still remains to be determined how TLR signaling is able to drive more rapid acidification kinetics. As can be seen from our analyses, not all phagosomes are proceeding to acidification at exactly the same rate, and there is still heterogeneity between a single genotype when individual phagosomes are monitored. One potential hypothesis is that assembly of the vacuolar ATPase on the phagosomal membrane is a TLR-dependent step. To assess this possibility, we can purify phagosomes from Wildtype and MyD88xTRIF-KO BMMs before and after stimulation with TLR ligands or *Salmonella* and blot for the V<sub>1</sub> and V<sub>0</sub> subunits of the vacuolar ATPase to determine if it is specifically assembled on phagosomal membranes after TLR activation. Similar results have been seen in DCs, so it is possible that the same mechanism functions in BMMs.

The TLR4-dependent cell death that we monitor in our studies is a pathway that many people have reported but is still not precisely dissected. Preliminary studies in our laboratory have shown that this cell death is Caspase-1 or Caspase-11 dependent. Furthermore, this cell death seems to specifically be TRIF-dependent, such that TRIF-KO animals are highly susceptible to *Salmonella* challenge (data not shown). We have generated chimeric TLR4 molecules expressed as transgenes in mice to study this question. Analysis of these mice (specifically a TLR4/5 chimera that recognizes LPS but signals through the TLR5 TIR domain and MyD88) may give us important clues as to how specific this death pathway is in certain cell types and if other TLRs are able to compensate for the role of TLR4 in different infection contexts.

Another experiment that could potentially expand our understanding of how *Salmonella* interacts with the innate immune system is to generate bone marrow chimeras with various TLR-knockout animals. For example, we can imagine that SPI-2 activation could be very important in bone marrow-derived cells but unimportant in the intestinal epithelium. In this scenario, transferring bone marrow from a MyD88xTRIF-KO animal

into Wildtype recipients should give us similar susceptibility as infection of a MyD88xTRIF-KO.

Along these same lines, we have been trying to determine the cell types in which SPI-2 activation is important. To do so we have generated several SPI-1 and SPI-2 effectors fused to cre recombinase. We are hoping to use these strains in vivo to infect flox-stop tdTomato mice and monitor active SPI-2 effector injection by tracking cells that have turned red. Preliminary in vitro studies suggest that this is a viable method for cell tracking, however, its efficacy in vivo is yet to be determined. Once this system has been optimized and these data are generated, more sophisticated mouse genetic techniques can be employed that would allow us to specifically turn TLR signaling on and off in the identified cell types.

### **Closing Remarks**

The data presented in this work have greatly contributed to our understanding of host-microbe interactions. Importantly, we are the first group to show that a bacterium can require signaling by the immune system to initiate its virulence. Interestingly, in the absence of TLR signaling, *Salmonella* is able to bypass the normal requirement for intracellular replication and replicate extracellularly without the need for SPI-2 induction. This is a very intelligent strategy on the part of the bacterium—to use as a contextual cue the product of the same pathway that will ultimately be evaded. By this mechanism *Salmonella* is able to maximize its fitness by not aberrantly expressing SPI-2 genes unless TLR signaling is actively occurring. Not only does this allow the bacterium to know that it has entered into the phagosome, but it also indicates to the bacterium that phagosomal survival is necessary. In other words, if TLR signaling does not occur, then *Salmonella* is able to replicate extracellularly, undetected. This interaction between the host innate immune system and pathogenic bacteria may occur with other bacteria as well, and a better understanding of these interactions could have profound implications for treating disease and increasing vaccine efficacy.

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