Innate Immune Control of *Salmonella enterica* Serovar Typhimurium: Mechanisms Contributing to Combating Systemic *Salmonella* Infection

Mary Jo Wick

Department of Microbiology and Immunology, Institute of Biomedicine, University of Gothenburg, Gothenburg, Sweden

Key Words

Salmonella • Innate immunity • Dendritic cell • Monocyte • Gut-associated lymphoid tissue

Abstract

Infections with *Salmonella enterica* serovars remain a serious problem worldwide. While serovar Typhi causes significant morbidity and mortality that is restricted to humans, serovar Typhimurium causes gastroenteritis in humans and can also infect other animals. As mice with the susceptible Nrp1 locus get systemic infection with serovar Typhimurium, murine infection models using this serovar have been widely used to decipher the immune mechanisms required to survive systemic *Salmonella* infection. This review summarizes recent studies in murine infection models that have advanced our understanding of the events that occur during the first days after oral *Salmonella* infection. The pathways of bacterial penetration across the intestinal epithelium, bacterial spread to draining (mesenteric) lymph nodes and dissemination to systemic tissues is discussed. The response of myeloid cell populations, including dendritic cells, inflammatory monocytes and neutrophils, during the early stage of infection is also discussed. Finally, the mechanisms driving recruitment of myeloid cells to infected intestinal lymphoid tissues and what is known about Toll-like receptor signaling pathways in innate immunity to *Salmonella* infection is also discussed.

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Introduction

Infection of humans and animals with the facultative intracellular pathogen *Salmonella* remains a serious global health problem. For example, the human-restricted serovars of *Salmonella enterica*, Typhi and Paratyphi, cause typhoid fever that claims several hundred thousand lives every year. This systemic disease, which is transmitted by contaminated food and water, is characterized by high fever, diarrhea and hepatosplenomegaly. Life-threatening complications of intestinal hemorrhage and perforation can arise and lead to sepsis, which is fatal if not treated. Nontyphoid serovars of *Salmonella* are also responsible for significant morbidity and mortality worldwide. Although gastroenteritis caused by nontyphoid serovars remains localized in healthy individuals, who typically recover without treatment, these infections can be fatal for immune-compromised individuals including those with HIV. Nontyphoid serovars also cause serious invasive diseases in children in developing countries.

Although *Salmonella* infection can be treated with antibiotics, these are often not readily available in endemic areas and antibiotic resistance is on the rise. Vaccines against typhoid fever do exist, but they exhibit only moderate protection in adults and are ineffective in children, and vaccines against nontyphoid *Salmonella* serovars currently do not exist. Overall, new and improved vaccines against *Salmonella* are clearly warranted. An important aspect of developing vaccines is to understand the pathogenesis of the infecting microbe. Indeed, infec-

tion models in mice are invaluable tools in the vaccine development process. In the case of *Salmonella*, infection of mice that have the susceptible allele of *Slc11a1* (*Nramp-1*) with serovar Typhimurium causes a systemic infection that is widely used to study the pathogenesis of and immunity to this bacterium. Although this mouse model, like any nonprimate model used to study human diseases, is not perfect, it has indeed provided valuable information regarding *Salmonella* infection. A clear advantage of murine models is that they allow mechanistic aspects of immunity to infection to be systematically studied. Indeed, such studies have contributed greatly to our understanding of the host response required to clear *Salmonella* infection, from the events that occur in the innate response to limit bacterial replication through development of adaptive immunity that ultimately eradicates the infection. This review will focus on the early events that occur during *Salmonella* infection to minimize bacterial growth and set in motion the adaptive immune response. The data discussed are from studies with *S. enterica* serovar Typhimurium in murine infection models and highlights recent findings. For the sake of brevity, *S. enterica* serovar Typhimurium is referred to as *Salmonella* in the remainder of this review.

The First Step of *Salmonella* Infection: Bacterial Penetration into Host Tissues

Physical barriers and resident immune cells in the intestine comprise the first line of defense against infection by orally acquired pathogens such as *Salmonella*. An important physical barrier preventing ingested bacteria from accessing host tissue is the single epithelial cell layer that separates the intestinal lumen from the underlying cells in the lamina propria. Several routes may contribute to *Salmonella* penetration across the intestinal epithelial layer [for review, see 1]. However, bacteria crossing the epithelium overlying Peyer's patches via M cells is a predominant pathway, particularly for invasive (pathogenicity island-1-proficient) bacteria. Bacterial crossing via M cells overlying solitary intestinal lymphoid tissues (SILTs), which are organized lymphoid tissue in the intestine other than Peyer's patches, also contributes to bacterial entry [2]. Finally, non-M cell-mediated 'cell sampling' routes whereby *Salmonella* can access the lamina propria have also been described [1, 3]. This pathway involves predominantly invasion-deficient *Salmonella* and is mediated by CX₃CR1⁺ phagocytes [1, 3]. Eloquent studies have greatly advanced our understanding of the function of

CX₃CR1⁺ phagocytes [for review, see 4] and interestingly, the CX₃CR1⁺ phagocytes involved in luminal sampling do not migrate to the mesenteric lymph nodes (MLN) that drain the intestine [5]. This suggests that bacterial transit mediated by CX₃CR1⁺ phagocytes may play a role in bacterial destruction rather than T cell priming. Alternatively, CX₃CR1⁺ phagocytes may pass bacteria or bacterial antigens to dendritic cells (DCs) for subsequent presentation to T cells. Thus, although the cell sampling pathway involving CX₃CR1⁺ phagocytes can contribute to penetration of invasion-deficient *Salmonella* into the lamina propria [3], M cell-mediated pathways dominate for invasion-proficient *Salmonella*.

Once invasive *Salmonella* use M cells to cross the epithelial barrier, the bacteria encounter resident cells in the subepithelial dome. This region is located beneath M cells in Peyer's patches and SILTs, and is rich in DCs, particularly of the CD8 α ⁻ subset [1, 6]. Moreover, although other myeloid lineage cells such as macrophages and neutrophils can be found in low numbers in Peyer's patches of naive mice using sensitive, high-throughput techniques such as flow cytometry [7, 8], cells expressing Gr-1 and F4/80 are not readily detected in the subepithelial dome by immunohistochemistry [1, 9]. Thus, the organization of Peyer's patches, with DCs being locally enriched in the subepithelial dome, allows these cells ready access to bacteria that penetrate via M cells. Given the pivotal role of DCs in priming naive T cells to start an immune response, DCs in intestinal lymphoid tissues are important players in initiating immunity to oral bacterial pathogens.

Dissemination of Orally Acquired *Salmonella* to MLN and Other Tissues

Once *Salmonella* penetrate the intestinal epithelial layer after oral infection they seed the intestinal lymphoid organs, Peyer's patches and SILTs, and the lamina propria. They also spread to the MLN draining the intestine, and to extraintestinal tissues, particularly the spleen and liver [2, 10–12]. Although much progress has been made regarding how *Salmonella* crosses the intestinal epithelium and establishes infection in the immediate target organs, our understanding of the mechanism by which *Salmonella* progresses to the MLN and extraintestinal tissues has lagged behind. However, progress in the field of migratory DCs [5, 13] and direct data addressing the dissemination of orally acquired *Salmonella* [12] have provided insight into this area. For example, Voedisch et al. [12] showed that colonization of the spleen and liver by

invasive *Salmonella* occurs by a mechanism distinct from colonization of MLN. In particular, increasing mobilization of intestinal DCs using a TLR agonist or a growth factor for DCs (FLT3L) increased *Salmonella* in the MLN but not the spleen or liver. Likewise, reducing CCR7-mediated DC emigration from the intestine via lymph by orally infecting CCR7-deficient mice reduced *Salmonella* in the MLN but not the liver or spleen [12]. However, some invasive *Salmonella* still reached the MLN in the absence of CCR7-mediated transport. This indicates that there are complementary, CCR7-independent mechanism(s) that can disseminate invasive *Salmonella* from the intestine to the MLN. Thus, the present picture of *Salmonella* dissemination supports an important role for CCR7-dependent bacterial transport from the intestine to the MLN via DCs migrating in lymph, while alternate pathway(s) allow the bacteria to reach systemic tissues. What are the pathway(s) that allow *Salmonella* to rapidly access extraintestinal tissues without necessarily relying on transport to the MLN in lymph-borne DCs? A possibility is that intestinal bacteria in phagocytes can directly access the blood, which can rapidly seed systemic tissues [14, 15]. Finally, data support an important protective role for MLN in limiting the spread of intestinal bacteria, both commensals [16] and pathogens [12], to systemic tissues. Thus, recent findings show specialized roles of intestinal cells and lymphoid tissues in controlling the dissemination of luminal bacteria, and that different processes dominate the colonization of the MLN and systemic tissues after oral *Salmonella* infection.

Dendritic Cell Responses to *Salmonella* Infection

The innate immune response is rapidly activated after oral infection, and significant changes in cell number are apparent within 2 days of intragastric *Salmonella* administration [7, 17]. Changes that occur include recruitment of DCs, inflammatory monocytes and neutrophils. Although DCs accumulate in Peyer's patches in response to infection, their increase is relatively modest [11]. Given the unique role of DCs in priming T cells and their limited role in keeping bacterial replication in check, it is likely that their functional features rather than their number per se is most important. For example, DCs expressing CCR6 are rapidly recruited to the sub epithelial dome region, and these cells have a key role in activating *Salmonella*-specific T cells [18]. In addition to the slight increase in DC number, another important aspect of the early DC response to infection is their maturation. This

process, which involves changes in DCs as detected phenotypically by increased surface expression of MHC class II and the co-stimulatory molecules CD86, CD80 and CD40, is necessary for efficient activation of naive T cells [19]. Given the role of intestinal DCs in maintaining intestinal homeostasis [20], the maturation process of intestinal DCs, perhaps coupled with recruitment of new 'nontolerized' DCs, is particularly important for their ability to prime naive T cells to orally acquired pathogens [19, 20]. Indeed, DCs harbor *Salmonella* after oral infection [11, 21] and are critical to priming bacteria-specific T cells [18]. Thus, although recruitment of CD11c^{hi} DCs to Peyer's patches during *Salmonella* infection is modest, the accumulation of particular subsets, such as DCs expressing CCR6 and/or cells less conditioned in a tolerogenic environment, may be important [20]. The initiation of DC maturation is an essential early event in the response to *Salmonella* infection and is a key step in starting an adaptive immune response to this pathogen.

Interestingly, *Salmonella* preferentially induces death of one subset of CD11c^{hi} conventional DCs during infection, the CD8 α ⁺ subset, while CD8 α ⁻ DCs and recruited inflammatory monocytes are resistant [22]. This bacteria-induced DC death depends on MyD88, an adaptor protein that controls signaling through many Toll-like receptors (TLRs), and on signaling through TNFR1. DCs have a fairly short life span of a few days, which could serve as a general mechanism to minimize undesired conditions such as chronic lymphocyte activation or autoimmunity. However, DCs must survive long enough to activate T cells, and an insufficiently short life span negatively influences the magnitude of the T cell response. What could be the consequences of infection-induced DC death? Is this a virulence factor aimed to undermine adaptive immunity and reduce the host's ability to clear the infection? Does it promote or impede bacterial spread? The answers to these questions are not clear, but we do know that conventional DCs are required to activate *Salmonella*-specific T cells in vivo [18]. The CD8 α ⁺ DC subset also has some special features of its antigen presentation capacity. For example, CD8 α ⁺ DCs efficiently take up dead cells, cross-present antigens on MHC-I and produce IL-12p70 and thus influence CD4⁺ Th1 T cell responses [23]. As IFN- γ -producing CD4⁺ Th1 T cells are critical to surviving *Salmonella* infection, [24], selective death of this subset could be a way for bacteria to avoid clearance. In this respect, it is also intriguing to note that recruited inflammatory monocytes have a limited capacity to process *Salmonella* for antigen presentation [7, 22]. However, CD8 α ⁻ DCs from infected mice have the capac-

ity to present *Salmonella* antigens on MHC-II for CD4⁺ T cell recognition [22]. The consequence of *Salmonella*-induced death of CD8 α ⁺ DC during infection is not known. However, it could be a way for *Salmonella* to slow down effective antigen presentation and dampen an effective Th1 T cell response, which may favor bacterial replication and spread within an infected host.

Dendritic Cell Maturation during Infection

As mentioned above, DCs in the steady-state intestine have an important role in maintaining tolerance to intestinal flora and potential food antigens, and the process of maturation is important in overcoming this tolerogenic bias. Infection with intestinal pathogens must therefore trigger DC maturation. How is DC maturation triggered *in vivo*? We know that an important initiating event occurs when conserved microbial structures, so-called microbe-associated molecular patterns (MAMPs) such as LPS and flagella, engage pattern recognition receptors on host cells. Indeed, host cells express different pattern recognition receptor families that recognize MAMPs, which act as common denominators indicating an infection. The receptor families involved in sensing bacterial infection include C-type lectin receptors, nucleotide-binding oligomerization domain-like receptors (NLRs) and TLRs [25–27]. Distinct functions of these receptor families create a complementary approach to detecting and controlling infections [25, 28]. For example, C-type lectin receptors often act as phagocytic receptors while NLRs are intracellular sensors of microbial components, particularly peptidoglycan structures, and also detect cell injury [25, 27]. Ligand recognition by NLRs results not only in production of cytokines and chemokines, but also in assembly of inflammasomes that results in production of the active forms of IL-1 β and IL-18 [25, 29]. The inflammasome is indeed important in the innate immune response to *Salmonella* [29, 30]. Recent reviews on NLR signaling and the inflammasome are available, and the reader is referred to these for more detailed discussion of these topics [25, 29].

TLRs are also important in controlling *Salmonella* infection [31]. A main function of TLRs, which are expressed by intestinal epithelial cells and immune cells, is to initiate signaling cascades that lead to pro-inflammatory cytokine and chemokine production by NF- κ B activation [26]. Several of the cytokines induced by bacterial triggering of pattern recognition receptors, particularly IL-1 β and TNF- α , can influence MHC-II and costimulatory molecule expression and contribute to DC maturation during

Salmonella infection [11, 21]. However, phenotypic maturation as assessed by altered expression of MHC and costimulatory molecules does not necessarily reflect functional maturation of the DCs. That is, full DC maturation must result in DCs that elicit effector functions in T cells (i.e. IFN- γ production) [32]. An important component of achieving fully functional DC maturation is signals delivered by direct DC interaction with microbial structures [32]. In contrast, signals delivered indirectly by cytokines in the absence of direct microbial contact results in phenotypic but not necessarily functional DC maturation [32]. Studies to decipher the contribution of direct versus indirect (cytokine-mediated) maturation of DC during *Salmonella* infection, which is biologically more complex relative to studies injecting purified bacterial products, have been performed [21]. Like studies using purified bacterial products, infection studies revealed that direct DC activation by *Salmonella* also influences phenotypic and functional DC maturation. However, differences in bacterial killing by DCs lacking TLR pathways that were used to remove the effect of pro-inflammatory cytokines (and thus indirect DC maturation) underscores the complexity of mechanistic studies in infection models [21]. Thus, DCs respond differently to direct bacterial contact relative to sensing cytokines in the environment. However, we know little about the role of direct versus indirect DC maturation during infection with a live pathogen, and given the complexity of studying cellular responses during infection with a replicating pathogen, understanding direct versus indirect DC maturation during infection requires further investigation.

Recruitment and Activation of Inflammatory Monocytes and Neutrophils

In contrast to the modest increase in DCs in response to oral *Salmonella* infection, neutrophils and inflammatory monocytes rapidly infiltrate Peyer's patches and mesenteric lymph nodes [1, 7, 11]. Inflammatory monocytes can be distinguished from conventional CD11c^{hi} DCs and other myeloid cells by, for example, their CD11c^{int}CD11b^{hi}Ly6C^{hi}Ly6G^{low} phenotype. Indeed, the accumulation of these cells in infected tissues results in their numeric dominance relative to neutrophils and DCs in the gut-associated lymphoid tissue during the acute phase of infection [7, 11]. Although the importance of bacterial uptake and killing by neutrophils during *Salmonella* infection has been known for some time, inflammatory monocytes are a relatively recently described myeloid lin-

eage cell whose function during infection is not fully understood. These cells, however, are predominant producers of products important in fighting bacterial infections, particularly TNF- α and iNOS, in infected tissues [1]. This feature also distinguishes them from conventional (CD11c^{hi}) DCs, which produce neither iNOS nor TNF- α in *Salmonella*-infected mice [7, 11]. The ability of inflammatory monocytes to produce TNF- α , iNOS and IL-1 β combined with their ability to take up and kill *Salmonella* [7], suggests that they contribute to holding initial bacterial replication in check. Although this may very well be the case, inflammatory monocytes are not as efficient at internalizing *Salmonella* as neutrophils [7]. Specific depletion experiments and cell type-specific ablation of effector functions, such as TNF- α and iNOS production, by inflammatory monocytes are required to fully elucidate their contribution to bacterial killing in vivo.

Another possible function of inflammatory monocytes during infection is to act as antigen-presenting cells. Indeed, inflammatory monocytes have several features that prompted investigation of their ability to activate T cells. For example, in addition to their ability to take up and kill *Salmonella*, they also have relatively high expression of MHC-II and co-stimulatory molecules, particularly CD80 [7, 11]. These are all important features of antigen-presenting cells. In addition, inflammatory monocytes express the integrin CD11c at intermediate levels, suggesting a phenotypic, and possibly functional, relationship to CD11c^{hi} conventional DCs. However, experimental data show that infection-induced Ly6C^{hi} inflammatory monocytes have a poor capacity to stimulate T cell proliferation when exposed to *Salmonella* ex vivo in an antigen-specific system [7, 22]. Inflammatory monocytes can, however, induce T cell proliferation when exposed to peptide and can stimulate T cell proliferation in a mixed lymphocyte reaction [1, 7]. Overall, the data thus far suggest a more likely role of inflammatory monocytes in containing bacterial replication rather than making a major contribution to activating bacteria-specific T cells during infection. Indeed, inflammatory monocytes may even inhibit or alter T cell function [33].

Mechanisms Driving Cell Recruitment to Intestinal Tissues during Infection

The inflammatory monocytes and neutrophils that accumulate in *Salmonella*-infected tissues described above are released into the blood early during the response to infection [7]. These cells enter Peyer's patches by a mech-

anism that can occur independently of the selectin MAdCAM-1 [7], which is required for T cell homing into Peyer's patches. Insight into the signals that drive monocyte and neutrophil homing to Peyer's patches and MLN during infection were provided by analyzing chemokine receptor expression on cells released into the blood in response to infection combined with kinetic studies of *Salmonella*-induced chemokine expression in Peyer's patches and MLN [7, 17]. Not surprisingly, the majority of circulating neutrophils expressed CXCR2 while circulating monocytes expressed a high level of CCR2 [7]. Consistent with this, CXCL2 and CCL2, the ligands for CXCR2 and CCR2, respectively, were significantly upregulated in blood, Peyer's patches and MLN already 48 h after infection [17]. Another chemokine that can participate in monocyte recruitment, CXCL9, was also upregulated rapidly in gut-associated lymphoid tissue after oral *Salmonella* infection. Interestingly, the follicle-associated epithelium and cells in the subepithelial dome were the primary initial sources of the neutrophil-recruiting ligand CXCL2. Consistent with their location, the ligand for attracting CCR6⁺ DCs to the subepithelial dome in response to *Salmonella* infection, CCL20, is also predominately produced by the follicle-associated epithelium [6, 17]. In contrast, localized production of the monocyte-recruiting chemokine CCL2 in Peyer's patches was not readily detected and instead an important source of CCL2 was the myeloid cells themselves in response to bacterial signals [17].

Triggering of pattern recognition receptors by their ligands is important in initiating a large number of events critical to fighting infection, and indeed TLR signaling and the inflammasome are important in controlling *Salmonella* infection [30, 31]. To further understand TLR signaling in the innate defense against *Salmonella*, the role of TLR signaling in myeloid cell recruitment and chemokine production was assessed in *Salmonella*-infected mice lacking one or more TLRs [17]. These studies revealed that complementarity in the TLR signaling pathways ensures recruitment of myeloid cells to infected tissues. That is, simultaneous removal of both MyD88 and TLR4, whose combined absence removes bacteria-mediated TLR signaling, was required to significantly decrease both monocyte and neutrophil influx into Peyer's patches and MLN. In contrast, removing signaling through TLR4 alone, removing flagella-mediated signaling by infecting wild-type mice with flagella-deficient *Salmonella*, or infecting TLR4 knockout mice with flagella-deficient *Salmonella* had no effect on monocyte or neutrophil recruitment to gut-associated lymphoid tissue, while MyD88 deficiency alone partially affected re-

cruitment [17]. In contrast, release of monocytes and neutrophils from the bone marrow into the blood was not significantly reduced even in infected MyD88^{-/-}TLR4^{-/-} mice [17]. The observed myeloid cell release into the blood in infected MyD88^{-/-}TLR4^{-/-} mice, and their reduced recruitment into intestinal lymphoid tissues in these animals, occurred despite severe reductions in chemokine expression in the tissues. This suggests that non-TLR-mediated mechanisms such as NLRs may contribute to the release of monocytes and neutrophils into the blood during infection and to the low level of recruitment into infected tissues. Alternatively, low TLR-mediated chemokine levels and/or redundant function of chemokines is sufficient to cause bone marrow release and low recruitment of myeloid cells into tissues during *Salmonella* infection. The signaling pathways and chemokines required to induce precursor release from the bone marrow, as well as those required for cell influx into infected tissue, are interesting areas that warrant further study.

Overall, TLR signaling is important from the host's perspective to survive infection. Given that a rapid and efficient response is required to contain infections, it is not unexpected that several complimentary and overlapping mechanisms, such as multiple receptors in the same family and distinct receptor families, are in place to ensure host survival. One such example is the multiple TLRs that sense bacterial infection and contribute to phagocyte recruitment to infected lymphoid tissues as described above. Such a finding is not surprising given the importance of recruited phagocytes in limiting bacterial replication and spread. Indeed, mice with defects in TLR signaling, and a corresponding impaired innate immune response, are more susceptible to infection [17, 31]. However, TLR signaling can also be exploited by *Salmonella* to enhance virulence. For example, *Salmonella*-induced TLR4 signaling impairs expression of homeostatic chemokines involved in organizing lymph node architecture, which in turn reduces the efficiency of the host's adaptive immune response and compromises bacterial clearance [34]. Furthermore, TLR signaling is necessary for acidification of *Salmonella*-containing vacuoles, which is required for induction of *Salmonella* pathogenicity island II and *Salmonella* pathogenicity island II-regulated virulence genes [35] that are critical for intracellular survival of *Salmonella*. That is, in the absence of TLR signaling (in mice with an Nramp-1-resistant background), *Salmonella* could not replicate intracellularly and thus had reduced virulence [35]. These recent data support a role for TLR signaling in *Salmonella* virulence, which adds a new layer to the role that TLRs play in host survival.

Concluding Remarks

Murine infection models have provided much insight into the initial events that occur after *Salmonella* infection and have paved the way for mechanistic studies allowing detailed understanding of the innate immune response to this oral pathogen. Indeed, recent elegant studies have shed light into the pathways of *Salmonella* penetration from the intestinal lumen and dissemination in the host, which have been experimentally challenging issues to address. Moreover, we are beginning to get a picture of the orchestration of different myeloid cell populations in responding to *Salmonella* infection, the mechanisms of their recruitment, and how they contribute to controlling bacterial replication and initiating adaptive immunity. However, there are still gaps in our knowledge, such as the mechanisms of functional DC maturation in vivo and how TLRs as well as other pattern recognition receptors contribute to recruitment and activation of innate cell populations. Moreover, despite a successful journey thus far in building a picture of how the host responds to combat *Salmonella*, this bacterium continues to reveal surprises. For example, recent studies have revealed how *Salmonella* exploits TLR signaling to reduce the host's adaptive immune response by impairing expression of chemokines involved in organizing lymph node architecture. TLR signaling is also required for *Salmonella* to express virulence genes when it resides in phagocytic vacuoles. Thus, the TLR system, which has traditionally been considered a host defense mechanism, also contributes to *Salmonella* virulence. This new twist of *Salmonella* pathogenesis highlights that the last word is not said regarding our understanding of the innate immune response to this formidable intracellular pathogen.

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