REVIEW

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Activation of *Salmonella*-specific immune responses in the intestinal mucosa

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Summary

The mammalian immune response to *Salmonella* has long been a subject of scientific study. Indeed, many of the general aspects of bacterial pathogenesis and host immune defense have been well described. However, a lack of clarity remains concerning important aspects of the host immune response to *Salmonella*, particularly with regard to the induction of an immune response in the intestinal mucosa. A major limitation has been the general lack of knowledge about specific antigenic targets that are recognized by both the innate and adaptive immune response in the intestine. Progress towards the identification of these targets is critical for the development of a detailed model of immunity to *Salmonella* and will lead to a better understanding of mucosal immune responses to other intracellular pathogens.

Key words: *Salmonella*, intestinal mucosa, immune responses

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INTRODUCTION

Salmonella enterica serovars *typhi* and *paratyphi* are the causative agents of human typhoid fever, a systemic disease of the reticulo-endothelial tissues that remains a serious health concern in developing countries. Current typhoid vaccines are only moderately effective, not suitable for use in the most vulnerable patients, or do not induce long-lasting immunity [75]. A typhoid-like disease caused by infection of susceptible inbred mice with *Salmonella enterica* serovar *typhimurium* (hereafter referred to as *S. typhimurium*) provides an excellent model system to increase our understanding of the immune response to human typhoid [66]. Natural infection with *Salmonella* is usually acquired enterally, before spreading to the spleen, liver, and bone marrow. Therefore, as with most microbial pathogens, contact between *Salmonella* and host immune defenses initially occurs across a mucosal surface. This review will focus on the induction of innate and adaptive immune responses to *Salmonella* in this particular environment.

ENTERIC INVASION AND SYSTEMIC DISSEMINATION OF *SALMONELLA*

As noted above, the natural route of *Salmonella* infection involves the gastro-intestinal tract (GIT), a heterogeneous tissue in terms of cellular composition, organization, and function. Although *Salmonella* can penetrate the intestine at almost any site in the GIT, the classical site of bacterial invasion is found in the Peyer's patches of the distal ileum and, to a lesser extent, the cecum [5]. Early studies noted that bacteria could be detected in the Peyer's patches 6 h after oral infection and in the draining mesenteric lymph nodes (MLNs) 24–48 h later [5]. As the infection progresses, increasing numbers of bacteria can be found in both the liver and spleen. Subsequent studies confirmed these observations using oral infection with either virulent or attenuated strains of *Salmonella* [23]. Therefore, the Peyer's patch is likely to be the initial site of *Salmonella* colonization and immune activation before dissemination to other lymphoid and non-lymphoid tissues occurs.

The Peyer's patches are specialized lymphoid aggregates found throughout the small intestine. Overlying the luminal surface of the Peyer's patch is the follicular associated epithelium (FAE), which can be distinguished from the surrounding villous epithelium by a lack of goblet cells, prominent brush border, and the expression of several digestive enzymes [49, 54, 55, 56]. Specialized epithelial cells called microfold (M) cells are found in the FAE and are characterized by the presence of pinocytic vesicles, flattened villi, and a flexible cytoskeleton [54]. M cells are highly endocytic and transcytose antigen from the intestinal lumen to invaginations at the basolateral surface, which can contain lymphocytes, dendritic cells (DCs), or other phagocytes [28, 53–56]. Beneath the FAE layer is the sub-epithelial dome (SED), a small area that contains DCs and macrophages and presumably serves to engulf antigen or microbial pathogens that penetrate the FAE. The SED overlies the major anatomical structures of the Peyer's patch, prominent B cell follicles and an intervening T cell area, termed the inter-follicular region (IFR).

Although M cells seem designed to continuously sample normal luminal antigens, they also serve as a portal of entry for *Salmonella* and many other viral and bacterial enteric pathogens [30, 56, 65]. Attachment of *Salmonella* to the luminal surface of an M cell is characterized by epithelial membrane ruffling, culminating in the active uptake of *Salmonella* by the host cell [13, 31]. The bacterial genes required for this process have been mapped to the *Ipf* fimbrial operon and *Salmonella* pathogenicity island 1. Mutants lacking these genes are unable to initiate attachment and invasion of M cells [3, 14, 18, 29, 36, 59]. Internalization of *Salmonella* can cause M cell destruction, creating discontinuity in the FAE and allowing further entry of bacteria and spread to neighboring lamina propria [30]. After transport across the M cell layer, macrophages and DCs in the SED can phagocytose *Salmonella* [24] and may also be induced by bacteria to undergo apoptosis [62, 65, 73]. The cell death induced by invasive *Salmonella* increases early colonization and is dependent upon caspase-1. Thus, caspase-1-deficient mice are more resistant to virulent *Salmonella* and have lower intestinal and systemic bacterial loads following oral infection [47].

After infection of the Peyer's patch, *Salmonella* can be cultured from the MLNs, spleen, and liver [5]. Presumably, bacteria exit the Peyer's patch via afferent lymphatics, arrive in the draining MLNs, and subsequently gain access to the blood and visceral organs via lymphatics and the thoracic duct. It seems likely that such bacterial migration through secondary lymphoid tissues would be mediated by circulating infected phagocytes that originate from the Peyer's patch, although this has not been formally demonstrated. Recently, an alternative model of *Salmonella* dissemination has also been described that places considerably less emphasis on the Peyer's patch. It was noted that non-invasive mutants of *S. typhimurium* retained the ability to infect mice and disseminate widely, being found within CD18-expressing phagocytes [74]. Thus, even in the absence of bacterial virulence factors that promote Peyer's patch invasion, *Salmonella* can still rapidly infect the murine host. Even more surprisingly, bacteremia was found to peak within 30 min after oral inoculation, indicating that this process of bacterial dissemination is remarkably efficient [74]. The terminal ileum contains a population of DCs that express the chemokine receptor CX3CR1 and are exclusively associated with the intestinal villi [57]. Recent data indicate that these DCs internalize *Salmonella* by extending processes through epithelial tight junctions and into the lumen of the intestine [57]. It seems likely that bacterial entry via this DC population could account for infection and dissemination associated with the alternative pathway of invasion, since Peyer's patch attachment and invasion is not required. However, the contribution of such a pathway to *Salmonella* infection and dissemination during natural infection has not been firmly established. Future experiments are needed to clarify the relative contribution of the classical Peyer's patch versus alternative pathways to *Salmonella* invasion in the intestine.

ACTIVATION OF INNATE IMMUNITY BY *SALMONELLA*

Activation of innate immune responses can occur after pathogen recognition by germ line-encoded pattern-recognition receptors (PRRs). At least two major families of PRRs are found in the intestine: Toll-like receptors (TLR) and the intracellular NOD receptor family [60]. *Salmonella* express a variety of different PRR-ligands, most notably lipopolysaccharide (LPS) and flagellin, which can activate TLR4 and TLR5, respectively [2, 21]. *Salmonella* also express peptidoglycans and muramyl peptides that have the potential to activate the intracellular receptors Nod1 and Nod2 [19, 20]. However, the exact contribution of each of these bacterial products to innate immune activation during *Salmonella* infection is not well defined. As intestinal epithelial cells are normally exposed to a wide variety of commensal enteric bacteria, mechanisms exist to keep unwanted innate activation tightly regulated [33]. For example, the expression of TLR4 appears to be limited to the basal cells of the intestinal crypt, an area that is inaccessible to bacteria under normal circumstances [58]. Given the initial attachment of *Salmonella* to Peyer's patches, it seems unlikely that LPS would be able to trigger innate inflammation through crypt TLR4 molecules, although this may occur during entry via the alternative pathway of infection.

The receptor for bacterial flagellin, TLR5, is expressed basolaterally on intestinal epithelial cells [16]. Invasive *Salmonella*, but not commensal bacteria, are able to transport flagellin to the basolateral membrane and activate nuclear factpor (NF)-κB expression and interleukin (IL)-8 production via TLR5 [16, 17].

Flagellin can also trigger the secretion of the chemokine CCL20, shown to be important for recruiting immature DCs [67]. Therefore it seems likely that flagellin is a major pro-inflammatory determinant of *Salmonella* during natural intestinal infection [79]. One note of caution is that many of the flagellin studies were performed *in vitro* using human model epithelial cell lines and require further validation *in vivo*. The generation of TLR5 gene-deficient mice should clarify the role of flagellin in the activation of intestinal innate immune responses during *Salmonella* infection. Furthermore, given the importance of the Peyer's patch to bacterial entry, it would also be interesting to examine TLR expression by M cells, DCs, and macrophages in the SED.

DCS, ANTIGEN ACQUISITION AND PRESENTATION *IN VIVO*

The uptake of antigen from the lumen to the intestinal mucosa is thought to be dependent on M cells [53–56]. M cells do not express MHC-II on their surface and hence are unlikely to be involved directly in antigen presentation to T cells. The SED of the Peyer's patch contains two main DC populations, conventional $CD11c^+$ $CD11b^{hi}$ "myeloid" DCs and $CD11c^+$ $CD11b^{lo}$ $CD8\alpha^-$ $B220^-$ "double negative" (DN) DCs [34]. The DN DCs are unique to the Peyer's patch and are the only DC subset actually found within the FAE [27]. The T cell IFR of the Peyer's patch also contains two populations of DCs: a similar DN DC subset to that found in the SED plus a $CD11c^+$ $CD8\alpha^+$ $CD11b^-$ "lymphoid" DC subset [27]. All three of these DC populations (myeloid, lymphoid, and DN) are capable of activating transgenic CD4 T cells *in vitro*, with the myeloid DCs being the most efficient [27]. However, this does not necessarily mean that all three populations actually present antigen to T cells during infection *in vivo*. Indeed, one study noted that only the DN and lymphoid DCs appear to acquire and present antigen to T cells during reovirus infection [12]. The Peyer's patch DC subsets involved in acquisition and antigen presentation during *Salmonella* infection are poorly defined.

Although *Salmonella* can infect DCs, it is not clear if they have a predilection for any of the DC subsets in the Peyer's patch, though studies using splenic DCs suggest that all DC subsets can be infected with *Salmonella in vitro* and *in vivo* [76, 78]. As noted above, virulent *Salmonella* can induce apoptosis of DCs and macrophages. In contrast, other studies have reported that virulent strains of *Salmonella* can inhibit antigen presentation by DCs without inducing cell death [6, 71]. If such DC inhibition and/or apoptosis occurs *in vivo*, it seems likely that uninfected DCs acquiring free *Salmonella* antigens or material from neighboring apoptotic cells are responsible for activating T cell responses in the Peyer's patch [25 , 62, 73, 77].

As noted above, *Salmonella* flagellin stimulates CCL20 secretion [67], which is the ligand for CCR6, a receptor that happens to be expressed specifically on SED "myeloid" DCs [7]. A plausible model therefore could be that uninfected SED $CCR6^+$ DCs migrate to sites of bacterial entry via detection of CCL20, become activated by exposure to flagellin, acquire *Salmonella* antigens, and then migrate to the IFR to activate naïve *Salmonella*-specific CD4 T cells.

M cells are thought to have receptors for sIgA [40] and can transport and deliver luminal antigen-sIgA complexes to Peyer's patch DCs [39, 61]. This may be an additional mechanism for DCs to acquire *Salmonella* antigens *in vivo*, especially during secondary infection. The presence of *Salmonella*-specific IgA in the intestine may serve to deliver antigen to Peyer's patch DCs more rapidly and therefore activate adaptive immunity more rapidly during secondary exposure. As such, the ability to induce *Salmonella*-specific mucosal IgA responses may be an important consideration in the design of novel *Salmonella* vaccines.

Outside of the Peyer's patch, sub-epithelial $CX3CR1^+$ DCs present in the villi can internalize *Salmonella* directly from the lumen [57], but it is not yet known if these DCs can activate *Salmonella*-specific T cells after migration to the MLNs. Interestingly, CX3CR1-deficient mice are more susceptible to *Salmonella* infection, suggesting that this DC population is critical to protective immunity. However, it is not yet clear whether this defect is actually due to a deficiency in *Salmonella*-specific T cell priming or to the defective recruitment and migration of other cell types, specifically inflammatory monocytes and macrophages during infection [15].

SALMONELLA-SPECIFIC T CELL PRIMING AND EARLY ACTIVATION

CD4 T cells are absolutely required for the control of both primary and secondary *Salmonella* infection [22, 51], while CD8 T cells appear to be more important for immunity to secondary challenge [38]. A major limitation to studying T cell activation in this model is that only three MHC class-II epitopes have been clearly defined [8, 46, 50]. All of the described epitopes are found within FliC and SipC proteins, both of which have been shown to be highly down-regulated in macrophage phagosomes *in vitro* [10] and may therefore not be representative of the endogenous memory response to *Salmonella*. In contrast, some newly discovered protective antigens are among the most highly expressed *in vivo*, although the T cell epitopes in these proteins have yet to be mapped [64]. However, at present the best tool available for examining *Salmonella*-specific CD4 T cell responses *in vivo* is a TCR-transgenic mouse called SM1 that is specific for flagellin (FliC 427-441) [45, 46].

Virulent *Salmonella* can activate adoptively transferred SM1 CD4 T cells in the Peyer's patch as early as 3–6 h after oral infection. SM1 T cells in the MLNs are activated by 9 h post infection, suggesting that the

Peyer's patch and MLNs are the initial sites of T cell priming after *Salmonella* infection [45]. However, recent experiments indicate that SM1 T cells do not become activated following infection with low oral doses of *Salmonella* [69]. It seems likely that such low doses are more representative of natural infection with *Salmonella* during typhoid fever, although to our knowledge this has not been empirically tested. Our interpretation of these low-dose experiments is that flagellin expression is likely to be rapidly down-regulated and may therefore not be an antigen target of CD4 T cells during a natural infection [10, 68, 69]. However, at present it remains the most thoroughly characterized candidate antigen discovered. Mapping other endogenous T cell targets recognized by the immune response to low-dose *Salmonella* infection should therefore be a high priority.

T CELL MIGRATION, EFFECTOR FUNCTION, AND MEMORY

Information on CD4 and CD8 T cell trafficking in the gut following an oral *Salmonella* infection is limited. Immunity to *Salmonella* during primary and secondary infection is clearly dependent on IL-12, interferon (IFN)-γ, and tumor necrosis factor α. [9, 37, 41–44, 51, 52], while the production of IL-10 and IL-4 may impair immunity to *Salmonella* [1, 11]. *In vitro* experiments comparing T cell activation by purified Peyer's patch or splenic DCs suggest that the cytokine environment in the Peyer's patch is suppressive or Th2 promoting [26, 32]. Recent data suggest that the intestinal epithelium may condition mucosal DCs via secretion of chemokines, so that these DCs are predisposed to drive Th2 responses [63]. Indeed, "myeloid" DCs in the Peyer's patch SED, being therefore anatomically close to the epithelial layer, secrete IL-10 and IL-4, while "lymphoid" DCs from the more distant IFR secrete more IL--12 [26, 27]. However, despite such mucosal priming nuances, the T cell response to oral or i.v. *Salmonella* infection is invariably a Th1 response, and a large population of IFN-γ-secreting effector T cells are generated [35, 68, 72]. It may be that the PPR-ligands produced by *Salmonella* override the mucosal bias for generation of Th2 responses in gastrointestinal lymphoid tissues.

The particular mucosal DCs involved in *Salmonella*--specific T cell activation may have a stronger impact on other aspects of T cell function. Recent data indicate that Peyer's patch DC activation leads to the expression of gut-homing molecules α4β7 and CCR9 on T cells, causing preferential migration to the intestine [4, 48, 70]. It seems therefore likely that migration patterns of *Salmonella*-specific T cells may vary considerably depending upon the route of infection. Preferential migration to intestinal non-lymphoid tissues would be unlikely to be advantageous during *Salmonella* infection, since this is not a major site of bacterial replication. However, the presence of an elevated frequency of memory T cells in the intestine may be important for defense against secondary infection. These issues have not been addressed in any detail in this particular model of infection.

CONCLUSION

A great deal remains to be unraveled about the induction of an immune response to *Salmonella* in the intestine. Detailed information about intestinal DC function, T cell priming, T cell migration, and the generation of T cell memory during typhoid are lacking. However, the identification of immune targets, combined with the use of antigen-specific tracking methodologies, should go some way to resolving these issues in the future. Understanding the induction of *Salmonella*-specific immune responses in a mucosal environment will be vitally important for the design and generation of more effective vaccines against typhoid fever.

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