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Protective host immune responses to *Salmonella* infection

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Abstract

Salmonella enterica serovars Typhi and Paratyphi are the causative agents of human typhoid fever. Current typhoid vaccines are ineffective and are not widely used in endemic areas. Greater understanding of host–pathogen interactions during Salmonella infection should facilitate the development of improved vaccines to combat typhoid and nontyphoidal Salmonellosis. This review will focus on our current understanding of *Salmonella* pathogenesis and the major host immune components that participate in immunity to *Salmonella* infection. In addition, recent findings regarding host immune mechanisms in response to Salmonella infection will be also discussed, providing a new perspective on the utility of improved tools to study the immune response to *Salmonella* infections.

Keywords

bacterial infection; B cells; CD4 T cells; immunity; protection

Salmonella is a bacterial genus within the Family Enterobacteriaceae that consists of a large group of genetically similar organisms with the ability to infect a large number of animal hosts [1,2]. The majority of clinical disease in animals and humans is caused by serovars within the *Salmonella enterica* subspecies and this can range from local gastroenteritis to a fatal disseminated disease. The exact clinical outcome of *Salmonella* infection depends largely on the individual serovar involved, the infected host species and the immunological status of the individual [1,2]. Some *Salmonella* serovars are able to infect a wide variety of mammalian hosts and are responsible for large outbreaks of gastroenteritis in the USA associated with contaminated meat, produce or processed food [3]. In contrast, *Salmonella* serovars Typhi and Paratyphi have a restricted host range and cause systemic disease in

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humans that can often be fatal [4]. *Salmonella* serovars that routinely cause gastroenteritis are also able to cause systemic disease in individuals with a primary or acquired immune deficiency. Indeed, *Salmonella* bacteremia is an emerging problem in Sub-Saharan Africa where it is associated with HIV, malaria or poor nutritional status [5]. Thus, a variety of *Salmonella* serovars are responsible for a wide range of disease in developed and developing nations.

Typhoid fever is caused by infection with serovar Typhi or Paratyphi and is responsible for approximately 21.7 million cases and 217,000 deaths annually [6]. Initial attempts to prevent typhoid involved the administration of inactivated whole-cell vaccines, but given the substantial frequency of side effects such vaccines are no longer available for use [7]. Currently, there are two commercial typhoid fever vaccines, an orally administered attenuated strain (Ty21a) and parenteral Vi capsular polysaccharide antigen. Ty21a and capsular polysaccharide antigen are currently licensed in many nations, including the USA, but are predominantly used as traveler's vaccines. Both of these vaccines suffer from moderate efficacy and are not widely used in typhoid endemic areas. Greater understanding of the host immune response to *Salmonella* infection is required in order to improve the efficacy of existing typhoid vaccines or develop new vaccines. Since serovar Typhi only infects humans, it is difficult to study this organism in laboratory animals. Aside from the recent use of humanized mouse models [8], there are no good *in vivo* animal models to examine host–pathogen interactions during Typhi infection. However, many investigators have examined the immune response to systemic *Salmonella* infection by using a mouse model of challenge with serovar Typhimurium [9]. Serovar Typhimurim-infected mice display many similar characteristics to typhoid fever patients in term of lesions in internal organs and bacterial distribution in tissues. Importantly, this model is distinct from the *Salmonella* colitis model where antibiotic-treated mice are challenged orally with serovar Typhimurium, causing diarrhea and intestinal inflammation [10]. In this review, information about *Salmonella* pathogenesis and immunity to *Salmonella* infection will be summarized largely from studies using the serovar Typhimurium-challenged mouse model.

Pathogenesis of *Salmonella* infection

Humans are typically infected with *Salmonella* after consuming food or drinking water contaminated with bacteria and the transmission of most serovars uses the fecal-oral route [11]. After oral ingestion of bacteria, *Salmonella* invade intestinal epithelial cells in the distal ileum [12]. In particular, *Salmonella* can target the specialized microfold cell (M cell) population overlying lymphoid structures called Peyer's patches (PPs) [13,14]. Although M cells are associated with PPs, they can also be found associated with smaller lymphoid aggregates known as solitary intestinal lymphoid tissues [15] and more rarely in the complete absence of defined lymphoid structures [16]. Although *Salmonella* normally enter the host through PPs, they can penetrate the intestinal epithelium at other locations where M cells are present [17], and also invade other (non-M cell) epithelial cells [18,19]. The ability of *Salmonella* to access intestinal epithelial cells is conferred by a collection of virulence genes encoded by *Salmonella* Pathogenicity Island 1 (SPI-1). Proteins encoded by SPI-1 form a needle-like Type III secretion system that allows the transport of several bacterial proteins into the host cell cytosol. These proteins induce changes in the host cells such as the

rearrangement of cytoskeleton and cell membrane and disconnection of epithelial cell junctions [12], facilitating *Salmonella* invasion [20]. After penetrating PP M cells, bacteria access the underlying structure of the lymphoid tissue which is an area rich in phagocytic cells and serves as the initial site of intracellular infection [21,22].

From the initial infection site in the PP, *Salmonella* can travel via the afferent lymphatics to the draining mesenteric lymph nodes (MLNs), and eventually gain access to the blood and systemic tissues via transit through efferent lymphatic vessels [23]. The transport of *Salmonella* from PPs to MLNs likely requires CCR7-dependent migration within $CD11c^+$ dendritic cells and one study has reported decreased bacterial loads in the MLNs of CCR7-deficient mice [24]. However, it is also possible that free bacteria can move through lymph to MLNs without help of immune cells [25]. After dissemination to systemic tissues, *Salmonella* replicate in phagocytes of the spleen, liver and bone marrow [25,26]. The cell types involved in the transport of *Salmonella* to these systemic tissues are still poorly understood. *Salmonella* can evade degradation in host macrophages by affecting the maturation of the phagosome and reducing the deposition of NADPH oxidase. This is achieved by a second Type III Secretion System encoded by *Salmonella* Pathogenicity Island 2 (SPI-2) [27,28]. In some studies, *Salmonella* have also been described to access dendritic cells (DCs) or $CD18^+$ intestinal phagocytes and subsequently disseminate rapidly to the blood in the absence of lymphatic access [29,30]. This alternative pathway could be important for rapid dissemination of bacteria but remains incompletely understood. Thus, although *Salmonella* initially enters the host via the intestinal mucosa, this organism can rapidly spread to systemic tissues. Any understanding of host immunity to *Salmonella* infection must therefore take into account the complexity of simultaneous mucosal and systemic immune responses to invading bacteria.

Immunity to *Salmonella* infection

Innate immune response to *Salmonella* infection

Salmonella initially interact with epithelial cells, which can recognize pathogenic bacteria and initiate an inflammatory response and recruit a variety of bone-marrow-derived phagocytes [31]. The early immune response to *Salmonella* in PP and MLNs involves the recruitment of neutrophils and inflammatory monocytes, and these responses are important for delaying the spread of bacteria to systemic tissues [25,32]. Indeed, neutropenia in HIV patients is a risk factor for bacteremia caused by nontyphoid *Salmonella* species [33]. Studies have also shown that neutrophil depletion increased the extracellular bacterial load of *Salmonella* within the liver microvasculature [34], suggesting that neutrophils effectively contribute to early defense and prevent bacterial dissemination. A recent study using the *Salmonella* colitis model also showed that neutrophils can be an important cellular source of IFN-γ in the intestinal mucosa during innate phase of serovar Typhimurium infection. Neutrophil depletion by anti-Ly6G antibody impeded early IFN-γ expression and reduced the severity of histopathological lesions during serovar Typhimurium infection [35]. Whether neutrophils are a major source of early IFN-γ in the absence of colitis remains to be determined. The conventional idea is that natural killer (NK) cells play a prominent role in producing IFN-γ during early stage of bacterial infection in the mouse typhoid model.

Indeed, a recent study has confirmed the role of NK cells, specifically Thy1+ NK cells, in producing IFN-γ during early serovar Typhimurium infection, suggesting a major role for this cell type in mediating IFN-γ-dependent control of serovar Typhimurium [36]. Since a subset of innate lymphoid cells express some NK cell markers, it is likely that at least a proportion of this early NK-derived IFN-γ actually derives from this innate lymphoid cell subset [37]. Together, these studies would support the involvement of a variety of innate cells in the early control of *Salmonella* infection through phagocytosis and the production of IFN-γ. During early *Salmonella* infection, inflammatory monocytes are also recruited and rapidly accumulate in infected PPs and MLNs where they can produce anti-microbial factors such as iNOS, TNF-α and IL-1β [32]. The recruitment of these phagocytes is mediated by local chemokines that are induced in a MyD88-dependent manner [38]. In addition, resident macrophages within the infected tissues can also phagocytose bacteria and recognize cytosolic fllagellin via the NLRC4 inflammasome complex to activate caspase-1 and induce pro-inflammatory cytokines IL-1β and IL-18 [39–41]. Resident DCs can also recognize *Salmonella* lipopolysaccharide and flagellin, which causes an increase in the expression of CCR7, CD80, CD86 and CD40 [42,43]. This maturation of the DC population enhances antigen presentation capabilities, and allows these cells to migrate to the T-cell area of the lymphoid tissue to initiate an adaptive immune response [44].

Early activation of T cells in the intestine

Antigen-specific reagents have recently been used to study the early activation of T-cell responses *in vivo*. This approach remains challenging for the *Salmonella* infection model since the frequency of the endogenous naive T-cell repertoire is very low and there is also a limited number of known *Salmonella* epitopes that are presented in MHC class-I and II molecules [45,46]. For this reason, several different T-cell receptor transgenic adoptive transfer systems have been used to monitor early CD4 T-cell activation in the intestine during *Salmonella* infection [47]. This approach involves transfer of naive T cells to elevate the frequency of antigen-specific T cells above the limit of detection for flow cytometry and immunohistological analysis. The first study of this kind in the *Salmonella* model described OVA-specific T-cell activation in response to systemic *Salmonella*-OVA infection [48]. A similar approach was successfully used to visualize the initial activation of OVA-specific T cells in the PP after oral infection [47]. However, a major limitation of this approach is that it involves visualization of host responses to a heterologous antigen that is over-expressed by bacteria, rather than an endogenous *Salmonella* epitope. To address this deficiency, a TCR transgenic adoptive transfer system that allowed *in vivo* tracking of CD4 T-cell responses to a natural I-$A^{[b]}$ epitope within *Salmonella* flagellin was developed [21]. Using this adoptive transfer model, CD4 T-cell activation was initially detected in PPs and then in the MLNs following oral infection. These *Salmonella*-specific CD4 T cells were activated to express surface CD69 within 3 h of oral infection and produced maximal levels of inter-leukin-2 (IL-2), 9–12 h later [21]. Additional studies using this model have demonstrated that $CD11c^{+}$ $CCR6^{+}$ dendritic cells play a key role in initiating the early *Salmonella*-specific T-cell responses within the PP [49]. This DC population is recruited to the follicle-associated epithelium in response to local production of CCL20 that is induced by innate host responses to *Salmonella* flagellin [44].

During this initial stage of T-cell activation, the draining MLNs also serve as an important site of *Salmonella*-specific T-cell activation. Indeed, activated *Salmonella*-specific T cells can be detected in MLNs within 9–12 h postinfection, demonstrating the remarkable efficiency of the initial immune response to *Salmonella* infection. At this early time point, the activation of *Salmonella*-specific T cells was not detected in any other secondary lymphoid tissue [21,49]. Additional studies point to an important protective role of the MLN during infection since removal of this lymphoid tissue correlates with increased bacterial loads and severe immunopathology in liver of infected mice [24]. It has also been reported that mice lacking MLNs are more susceptible to relapsing typhoid following antibiotic treatment, further suggesting the host response within MLNs functions to inhibit the dissemination of bacteria [50].

Protective immunity to infection

A protective role for CD4 T cells in primary bacterial clearance has been established by infecting immune deficient mice with attenuated *Salmonella*. Mice lacking a thymus, αβ T cells, MHC class-II or T-bet+ Th1 cells, are unable to resolve this infection [51–54]. In marked contrast, mice that lack γδ T cells or B cells are able to resolve primary infection with attenuated *Salmonella* [55–57]. Some studies have shown that β2μ-deficient mice that lack surface MHC class-I can resolve primary infection with attenuated *Salmonella*, suggesting that MHC class-I-restricted CD8 T cells are not essential for host immunity [52]. However, these β2μ-deficient mice also lack expression of nonclassical MHC molecules and CD1, and may also be able to express free MHC class-I molecules in the absence of β2μ. Recent experiments using mice that only lack MHC class-I or cytotoxic granules suggest a modest protective role for CD8 T cells during the resolution of primary infection [58]. Together, these data point to a combined role for CD4 and CD8 T cells in the resolution of primary infection.

After clearance of primary infection, mice develop robust protective immunity to secondary challenge [59]. Studies examining this acquired immunity again suggest an important role for CD4 and CD8 T cells in bacterial clearance. However, the robust protective immunity observed in these mice cannot be transferred to naive mice by adoptive transfer of spleen cells, but required the addition of immune serum [60]. In agreement with these data, mice lacking B cells were able to control primary infection with an attenuated strain but succumbed to rechallenge with virulent *Salmonella* [55–57]. Although these data suggest that antibody can contribute to protective immunity during secondary infection, a recent study comparing B-cell-deficient mice (JhD) with transgenic mice containing B cells that are unable to isotype switch or secrete antibody suggests that B cells can play an additional protective role even in the absence of antibody secretion [61]. Indeed, B cells have previously been reported to function as antigen presenting cells and an important source of inflammatory cytokines during *Salmonella* infection [55,62–63]. Since the initial proliferation of *Salmonella*-specific T cells involves interaction with DCs in the T-cell area of lymphoid organs [21,47], a role for B cells in antigen presentation is likely to require secondary signals after this initial expansion has occurred. The details of how such antigen presentation would occur are unclear, especially since germinal center formation is significantly delayed in *Salmonella*-infected mice [64]. Although these data point to a

protective antibody-independent role for B cells, serum transfer experiments confirm that *Salmonella*-specific antibody can also be protective in some circumstances. This protective effect of antibody may be due to complement fixation or opsonization of free bacteria [65] but could also involve indirect effects on T-cell activation due to enhanced antigen presentation of opsonized bacteria [66]. Altogether, these studies suggest a central role of CD4 T cells in acquired immunity to *Salmonella* infection with an additional important contribution from both CD8 T cells and B cells.

Recent studies have attempted to characterize the protective CD4 and CD8 T-cell response in more detail. Several studies have suggested an inordinately large degree of CD4 clonal expansion after initial infection with *Salmonella*, such that over 50% of peripheral T cells show some evidence of activation and the acquisition of effector function [67–69]. These expanded T-cell populations also gain the capacity to migrate to nonlymphoid tissue sites of bacterial replication such as liver [70]. Thus, a large population of *Salmonella*-specific effector T cells is generated after infection and some of these cells access nonlymphoid tissues, presumably to control bacterial replication. A recent study has examined the stability of immune memory after primary infection and suggested that *Salmonella*-specific CD4 T cells were stable for more than one year after initial oral infection. This stability appears to be maintained by low-level peptide:MHCII presentation by persistently infected phagocytes to a small number of CD4 T cells in the secondary lymphoid organs that contained bacteria [71].

It is clear that the development of Th1 cells is required for bacterial clearance since mice lacking T-bet, IFN-γ or IFN-γR are unable to resolve *Salmonella* infection. However, there are additional data to suggest that Th17 cells can play an important protective role in the *Salmonella* model. Both IL-17 and IL-22 are produced in the intestinal mucosa early after oral *Salmonella* infection [72]. Although these cytokines can be produced by other cell types, *Salmonella*-specific Th17 cells have also been detected in mucosal tissues [73]. After *Salmonella* infection, IL-17A-deficient mice demonstrate a modest increase in bacterial dissemination, suggesting that IL-17 contributes to the maintenance of the mucosal barrier [74]. Indeed, the depletion of intestinal CD4 T cells that accompanies simian immunodeficiency virus infection selectively blunted the intestinal IL-17 response in rhesus macaques, allowing increased translocation of *Salmonella* to the mesenteric lymph nodes and spleen [75]. The production of intestinal IL-22 also induces intestinal epithelial cells to produce antimicrobial peptides such as lipocalin-2, an antimicrobial protein that prevents bacterial iron acquisition and is active against luminal bacteria. However, Serovar Typhimurium also possesses virulence genes involved in the biosynthesis and uptake of salmochelin which confers lipocalin-2 resistance [76,77]. Thus, although Th1 cells are critical for bacterial clearance in systemic tissues, Th17 cells most likely play an important additional protective role in preventing bacterial dissemination from the intestine.

Noncognate T-cell stimulation during *Salmonella* infection

During *Salmonella* infection, the expanded pool of responding *Salmonella*-specific CD4 T cells is able to relocate to infected tissues and secrete effector cytokines. Recent studies suggest that the elicitation of this effector response can occur in response to noncognate

stimuli in addition to direct cognate TCR stimulation (Figure 1). Noncognate activation of T cells has been studied extensively for virus-specific CD8 T cells and has been shown to involve inflammatory cytokines such as IL-12 and IL-18 [78–80]. In addition, a recent study has shown that OVA-specific memory CD8 T cells can be stimulated via noncognate signals during *Salmonella* infection. This mechanism was observed to require NLRC4 inflammasome activation and IL-18 release by $CD8\alpha^+$ DCs [81]. During bacterial infections, a variety of inflammatory cytokines such as IL-1β and IL-18 are produced in infected tissues as a result of host recognition of microbe-associated molecular patterns via multiple innate immune receptors including Toll-like receptors (TLRs) and inflammasome components [82]. Thus, an inflammatory environment may exist within *Salmonella*-infected tissues that allow CD4 T-cell effector functions to be elicited in the absence of TCR stimulation. It has been shown that *Salmonella*-specific CD4 T cells produce IFN-γ immediately after injection of lipopolysaccharide [83] and our laboratory has recently demonstrated that this response can also be induced by a variety of TLR agonists [84]. This innate T-cell response to a TLR agonist required the inflammsome components NLRC4 and NLRP3 and resulted in the production of IL-18. *Salmonella*-specific Th1 cells were rapidly activated to produce IFN-γ in an IL-18R-dependent manner and the absence of this response delayed bacterial clearance. This pathway of innate stimulation of effector T cells may effectively lower the threshold for CD4 T-cell activation in infected tissues, thus amplifying the host response to infection. Also, this mechanism raises the question of whether *Salmonella*-specific Th1 cells can be stimulated in a noncognate manner in the context of super-infection or co-infection with a related pathogen. The development of a co-infection model utilizing *Salmonella* and another pathogen may therefore be useful to examine the contribution of *Salmonella*-specific Th1 cell in the clearance of other infections. It could be possible that the reduced threshold for CD4 T-cell stimulation may confer an advantage of host immune system to response rapidly to multiple infections, which may occur under natural circumstances, providing cross-protection to other pathogens.

Conclusion

Salmonella bacteria have evolved mechanisms to evade immune defense and cause chronic infection in the host. The host immune response involves innate and adaptive components that are differentially active in mucosal and systemic lymphoid tissues. CD4 T cells have been shown to play a major role in protective immunity during primary and secondary Salmonella infection. These CD4 T cells are activated initially in the PP and MLN after oral infection, before additional stimulation occurs in systemic tissues. In addition to CD4 T cells, innate immune cells, CD8 T cells and B cells all make an important contribution to pathogen clearance. Recent studies examining noncognate stimulation of *Salmonella*-specific T cells suggests that expanded CD4 and CD8 effector T cells can acquire the capacity to rapidly respond to inflammatory cues, thus reducing the threshold for stimulation in infected tissues. These new findings suggest that effector T cells might be activated in a largely nonspecific manner and the development of a co-infection model may be useful to unravel this response. Future work should allow greater understanding of the induction, maintenance and stimulation of *Salmonella*-specific effector cells and lead to the development of improved vaccines for typhoid.

Future perspective

Although considerable progress has been made in our understanding of the host immune response to *Salmonella* infection in the murine typhoid model, some important questions remain to be addressed. First, the overall relevance of this mouse model to human infection requires additional clarification. In particular, it is not yet clear if the mouse model of infection with serovar Typhimurium effectively models typhoid or systemic nontyphoidal Salmonellosis. Recent studies have found overlap between antigens targeted in mouse infection, children suffering from nontyphoidal Salmonellosis and experimental infection in volunteers [85,86], providing some hope that mouse studies represent a viable preclinical model. However, the development of a murine model that is permissive for *S.* Typhi infection would still be an important future goal. Second, although we have a broad understanding of the different arms of the immune response that confer protective immunity in the murine model, we have a very rudimentary understanding of the target antigens recognized by these protective responses. This issue is important to resolve because greater definition of antigen targeting could lead to the development of a new sub-unit vaccine or important gains in the immunogenicity of live attenuated vaccines for humans.

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EXECUTIVE SUMMARY

Pathogenesis of *Salmonella* infection

- *Salmonella* infect via Peyer's patches and invade underlying tissue.
- *Salmonella* gain access to systemic tissues.
- SPI1 and SPI2 Type III Secretion Systems are integral to *Salmonella* virulence.

Immunity to *Salmonella* infection

- **Innate immune response to *Salmonella* infection**
- Initial responses involve recruitment of phagocytes and IFN-γ production.
- Neutrophils and NKs cells are the main source of early IFN-γ.
- Recruited inflammatory monocytes produce iNOS and cytokines.
- **Early activation of T cells in the intestine**
- TCR transgenic mice have been used to visualize early T-cell activation in the intestine.
- CD4 T-cell activation occurs within hours of oral infection.
- Mucosal dendritic cells drive early clonal expansion of T-cell responses.
- **Protective immunity to infection**
- CD4 and CD8 T cells combine to resolve primary infection.
- B cells are critical for secondary protective immunity.
- CD4 Th1 cells and Th17 cells collaborate to combat infection.
- **Noncognate T-cell stimulation during *Salmonella* infection**
- Th1 cells can be stimulated by cognate and noncognate pathways.
- IL-12 and IL-18 are critical to induce a noncognate response.
- Noncognate T-cell stimulation provides nonspecific protection.

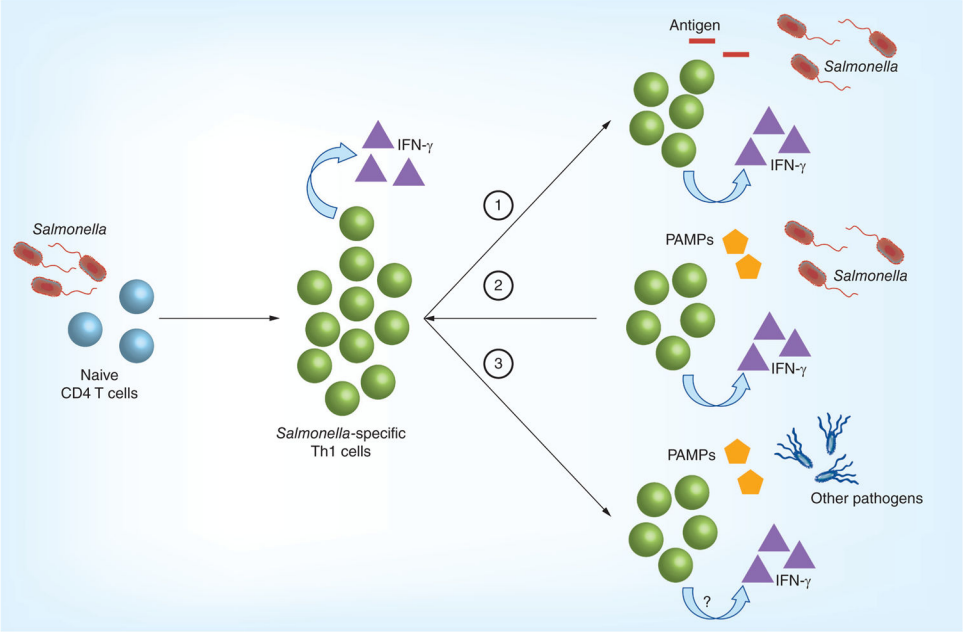

Figure 1. The role of noncognate T-cell activation in *Salmonella* immunity

During primary infection of *Salmonella*, dendritic cells present antigens to naive CD4 T cells causing expansion of a pool of *Salmonella*-specific Th1 cells. These Th1 cells can acquire the ability to produce the effector cytokine IFN-γ and migrate to infected tissues where they can be stimulated in a cognate fashion through TCR ligation by MHC:peptide complexes expressed on infected cells (top, antigen/*Salmonella*). In addition, these Th1 cells have the capacity to be stimulated by signals elicited by PAMPs (middle, PAMPs/salmonella) and may also be able to respond to co-infection with other pathogens (bottom, PAMPs/other pathogens).