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Immunity to intestinal pathogens: lessons learned from *Salmonella*

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Summary

Salmonella are a common source of food or water-borne infection and cause a wide range of clinical disease in human and animal hosts. *Salmonella* are relatively easy to culture and manipulate in a laboratory setting, and the infection of laboratory animals induces robust innate and adaptive immune responses. Thus, immunologists have frequently turned to *Salmonella* infection models to expand understanding of immunity to intestinal pathogens. In this review, I summarize current knowledge of innate and adaptive immunity to *Salmonella* and highlight features of this response that have emerged from recent studies. These include the heterogeneity of the antigen-specific T-cell response to intestinal infection, the prominence of microbial mechanisms to impede T and B-cell responses, and the contribution of non-cognate pathways for elicitation of T-cell effector functions. Together, these different issues challenge an overly simplistic view of host-pathogen interaction during mucosal infection but also allow deeper insight into the real-world dynamic of protective immunity to intestinal pathogens.

Keywords

bacterial immunity; $CD4^+$ T cells; lymphocyte tracking; non-cognate activation; immune evasion

Introduction

Immunity to intra-macrophage pathogens requires an infected host to generate a robust and sustained CD4 Th1 response. The development of this response is best studied in animal models where Th1 cells are required for macrophage activation, such as *in vivo* models of *Salmonella*, *Leishmania*, and *Mycobacteria* (1–3). Each of these infection models has been utilized over the years to reveal important aspects of CD4 T cell biology (1, 3, 4). Laboratory infection of mice with *Salmonella* is a particularly attractive model since *Salmonella* are easily cultivated and manipulated genetically, this organism is relatively safe to use in the laboratory, and a the natural oral route of infection can be utilized (5). As might be expected, *Salmonella* infection of inbred mouse strains induces a robust $CD4^+$ T-cell response that is essential for protective immunity to secondary infection (6–10). More

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surprisingly for an intra-macrophage pathogen, *Salmonella* also induces $CD8^+$ T-cell and antibody responses that can contribute to the resolution of infection (8, 10, 11). Thus, the *Salmonella* infection model is highly amenable to laboratory study and allows the direct examination of protective $CD4^+$ Th1 cells with the additional ability to examine the role of $CD4^+$ T cells in the generation of protective $CD8^+$ T-cell and B-cell responses. The only real limitation to using the *Salmonella* mouse model has been the relatively poor characterization of antigen specificity and a corresponding paucity of antigen-specific detection reagents (12). However, in the last decade, this problem has largely been overcome, and there are now antigen-specific reagents available for studying *Salmonella*-specific $CD4^+$, $CD8^+$, and B-cell responses *in vivo* (13). Recent studies have used these reagents and uncovered an unappreciated complexity in the generation, function, and maintenance of $CD4^+$ Th1 cells during *Salmonella* infection. This review summarizes current understanding of host immunity to *Salmonella* infection and subsequently discusses recent studies of interest to host-pathogen interactions during mucosal or systemic infection.

Classification

Salmonella belong to the *Enterobacteriaceae* family of Gram-negative bacteria, which includes several medically important pathogens, such as *Shigella*, *Yersinia*, and *Klebsiella*. The *Salmonella* genus consists of only two species, *S. enterica* and *S. bongori*, and almost all *Salmonella* infections of humans and animals are caused by a single sub-species of S. *enterica*, called *S. enterica enterica* (14). This sub-species contains over 2000 genetically similar serovars that are often grouped according to their ability to cause local or systemic disease in different animal hosts. *S. enterica enterica* serovars, such as Typhimurium and Enteritidis, are generalists that can typically cause gastroenteritis in humans and a wide range of animal species (15). Although they normally cause a localized infection of the intestine, these serovars can also be responsible for severe systemic disease in an immune-deficient host (16). A second group of *S. enterica enerica* serovars, such as Dublin (bovine) and Cholerasuis (swine), display a more restricted pattern of infection that is normally associated with certain host species but are more likely to cause systemic disease in the presence of a functioning immune system (15). Lastly, there are a small number of *S. enterica enterica* serovars that display a highly restricted pattern of infection in a single species, but are associated with serious systemic infections (17, 18). The most prominent of these highly restricted serovars is *S. enterica enterica* serovar Typhi, which causes typhoid fever in humans but is unable to infect any other mammal (19).

In developed nations, human infections with *Salmonella* are often observed as large outbreaks of rapid-onset gastroenteritis caused by the contamination of meat, produce, or processed food with serovars that can come from a variety of different animal reservoirs (20). The health and economic impact of these food outbreaks are substantial and *Salmonella* infections remain the largest cause death due to food-borne contamination in the US (20, 21). The pattern of *Salmonella* infections in many developing nations can be markedly different, especially if there is a limited sanitation infrastructure that allows human-to-human transmission to occur. In this environment, the human-restricted serovars S. Typhi and S. Paratyphi can thrive and cause typhoid fever, a systemic disease that is transmitted between infected humans without an animal host (19). The incidence of Typhoid

fever is difficult to calculate but has been estimated at 21.7 million infections and 217,000 deaths annually (18, 22). Unlike the rapid-onset gastroenteritis commonly observed in developed nations, the symptoms of Typhoid fever can take weeks to develop following the ingestion of contaminated food or water, but, since this is a systemic infection, a fatal outcome is more likely (18, 22, 23). As noted above, non-typhoidal *Salmonella* serovars that normally cause gastroenteritis can also cause serious systemic infections in immune-deficient individuals (16, 24, 25). Thus, rare patients with primary genetic deficiencies different cytokines or receptors associated with Th1 or Th17 responses can develop repeated and/or systemic infections with *Salmonella* serovars that normally cause acute gastroenteritis (16). A more widespread problem is the incidence of systemic Salmonellosis caused by non-typhoidal *Salmonella* infections in young children, HIV positive individuals, or patients who happen to be co-infected with malaria (25, 26). In these individuals, an underlying immune suppression or developmental delay in immune competence allows systemic infection to occur. Indeed, non-typhoidal salmonellosis (NTS) infections have been recognized as an emerging cause of bacteremia in sub-Saharan Africa (27). In summary, *Salmonella* remains an important cause of gastroenteritis in developed nations due to the continued prevalence of food-borne outbreaks, while developing nations deal with more serious systemic illnesses associated with typhoidal or non-typhoidal serovars.

Animal models for studying *Salmonella* infection

Given the broad host range of many *Salmonella* serovars, almost any warm-blooded animal could be considered as an animal model of human infection. However, due to economic considerations and the availability of immunological reagents, inbred mice are most commonly used to study *Salmonella* (1, 5). Since *Salmonella* infection of mice usually results in systemic infection without diarrhea or infection of the intestinal *lamina propria* (28), gastroenteritis is sometimes studied using a calf intestinal loop model that allows direct examination of the intestinal inflammation and fluid secretion (5, 29). An alternative approach is to pre-treat mice with antibiotics, which reduces the intestinal microbiota prior to *Salmonella* infection (30). By removing the natural colonization resistance mediated by host microbiota, *Salmonella* are able to induce an inflammatory colitis, and this simple model allows for mechanistic study of intestinal pathology and *Salmonella* growth in the intestinal lumen (30, 31). Without the use of antibiotics, oral *Salmonella* infection of mice typically causes a systemic infection that is considered a useful model of human typhoid fever (1, 5, 29). In both mouse and human typhoid, bacteria enter the host by specifically crossing the epithelial cells overlying Peyer's patches and then transit to the spleen, liver, and bone marrow, where they replicate in the reside phagocyte reservoirs at these sites (32, 33). As with human typhoid, *Salmonella*-infected mice develop profound hepato-splenomegaly and susceptible mice die from systemic infection (1, 5, 34). Despite the many similarities between human and murine Salmonellosis with respect to the route of entry, tissue tropism, cellular location of bacteria, and major immune responses, there are also some important differences. The *Salmonella* serovars that are used to infect mice are genetically distinct from the serovars that cause typhoid in humans and most notably lack expression of the Typhoid toxin and the Vi Capsule Polysaccaride (ViCPS) (35, 36). The lack of a capsule in serovars used for mouse infections probably explains the lack of any

'stealth' incubation period before the clinical signs of infection occur. Indeed, Vi CPS expression can reduce TLR-dependent inflammatory responses and prevent complement deposition during *Salmonella* infection (37, 38). Although serovar Typhi does not infect mice, recent studies have shown that humanized mice are permissive for infection with this serovar (39–41), and this opens up an important new avenue to study immunity to typhoid. However, given the cost and time required to generate humanized mice, it is unlikely that this new model will replace simple infection of inbred mouse strains with non-typhoidal serovars. TLR11-deficient mice have also been examined as a possible model of serovar Typhi infection (42); however, this model has proved difficult to reproduce in our laboratory. Therefore, the vast majority of laboratory studies examining immunity to *Salmonella* involve infection of inbred mice with non-typhoidal serovars.

Inbred strains of mice can be highly resistant or susceptible to laboratory *Salmonella* infection. Resistant strains of mice express a protective allele of the Slc11a1 gene that encodes an ion transporter important for resolving primary challenge with serovar Typhimurium (43). Since these mouse strains can survive infection with virulent bacteria, they are often used to study the resolution of a primary infection, effector T-cell responses, bacterial persistence, and the establishment of immune memory (6, 44–46). Indeed, the protective allele of Slc11a1 has now been backcrossed to the susceptible C57BL/6 strain to facilitate the examination of these issues using gene-deficient mice (47). However, from an immunological perspective, there is one crucial caveat to the use of resistant strains. Many studies have shown that protective immunity to *Salmonella* on a resistant background is largely mediated by antibody, rather than $CD4^+$ T-cell responses (48, 49). Actually, this limitation of the model is actually an important feature, since there is considerable evidence that antibody responses can be protective during typhoid and non-typhoidal disease in humans (50–53). However, any examination of *Salmonella*-specific $CD4^+$ T-cell responses in resistant mice must be made with the knowledge that this particular immune response is non-essential for bacterial clearance. While this issue can be largely irrelevant for general studies on T-cell biology, it becomes more important if the overall goal is to study the protective role of *Salmonella*-specific T cells *in situ*.

Inbred strains of mice that express a mutant allele of the Slc11a1 gene are extremely susceptible to *Salmonella* infection and do not remain alive long enough to provide a useful model for the study of protective responses against virulent strains (28). As might be expected, studying pathogen-specific $CD4^+$ T-cell responses is challenging in a model where mice develop fatal infection with 1–10 bacteria. However, by using bacterial strains with reduced virulence or by focusing on acquired protective immunity after vaccination, a great deal can be learned about the protective function of $CD4^+$ T cells in this model (8–10). Thus, *Salmonella* infections of resistant or susceptible inbred strains of mice are both viable approaches for studying the immune response to infection. The use of either model usually depends on whether the overall goal is to understand the role of $CD4^+$ T cells or antibody in protective immunity, the dynamics of bacterial persistence, or the development of immune memory.

Innate immune response to *Salmonella*

Salmonella infection of mice induces a robust innate immune response that involves epithelial cells, local phagocyte populations, and potentially memory T cells in the intestine (54). Many of these host cells can directly detect *Salmonella* products, such as lipopolysaccharide (LPS) lipid A via TLR4, flagellin via TLR5, and bacterial amyloid fibers via TLR1/2 (55–57). Recognition of bacterial ligands by TLRs increases the bactericidal activity of local tissue macrophages, induces the maturation and migration of dendritic cells, and initiates the production of inflammatory cytokines and chemokines (54). NOD-like receptors (NLRs) are also critical for the induction of inflammatory responses, since they can detect the translocation of bacterial products into the cytosol of epithelial cells (58, 59). As *Salmonella* inject bacterial proteins into epithelial cells as a virulence strategy to induce initial uptake, NLR-dependent cytosolic detection provides an important mechanism for the host to discriminate between commensal bacteria and *Salmonella* (60).

The early innate response to infection involves rapid recruitment of neutrophils and monocytes to intestinal lymphoid tissues and these cell populations congregate into inflammatory foci around infected cells (61, 62). Recruitment of monocytes and neutrophils occurs normally in the absence of TLR4 and TLR5 (62), suggesting that there is significant redundancy in the initial TLR and NLR-dependent responses that contribute to early recruitment of inflammatory cells. These migrating monocytes and neutrophils increase the local production of TNF-α, IL-1, and nitric oxide, which is likely to be important for restraining initial bacterial replication (61). While inflammatory monocytes can acquire expression of CD11c, high levels of MHC class I, and function as effective antigen-presenting cells (63), the role of these cells in intestinal T-cell activation has not been completely resolved. While an early role for monocyte-derived DCs has been found to be essential for Th1 development during *Salmonella* infection (64), it has also been shown that *Salmonella* can effectively block monocyte maturation (65, 66). A variety of other inflammatory cytokines are produced as a consequence of the innate response in the intestine, including IL-6, IL-12, IL-18, and IL-23, which together are important for providing an environment that allows differentiation of $CD4^+$ T cells towards both Th1 and Th17 linages (31). Indeed, as discussed below, a mixed Th1 and Th17 $CD4^+$ T-cell response has been detected in the intestine of *Salmonella*-infected mice (67). NK cells are another important contributor to the early innate response to *Salmonella* infection and indeed are probably more important that T cells in controlling early bacterial growth (68, 69). Recent examination of the role of NK cells in *Salmonella* infection has confirmed a protective role for NK cells that is largely mediated by the production of IFN-γ (70). Importantly, these studies also suggested that NK cells can participate in secondary responses during vaccine-mediated protection and that different live attenuated vaccines can vary in their ability to elicit this protective effect (70). Further studies are required to examine the contribution of NK cells during secondary responses and whether this reflects a direct effect of NK cells or an indirect effect on responding Th1 cells.

T-cell response to *Salmonella* infection

Infection of susceptible mice with *Salmonella* infection is the best of the available models to study *Salmonella*-specific $CD4^+$ Th1 responses since these cells actively participate in bacterial clearance. The inherent problem of using highly susceptible mouse strains has been addressed by (i) examining immunity to *Salmonella* strains with reduced virulence, (ii) examining the secondary response to fully virulent bacteria after vaccination, and (iii) examining immunity with the use of antibiotics to resolve primary infection. These different approaches fit well with the long history of research in developing attenuated *Salmonella* strains as vaccine vectors (71). We summarize the main findings from each of these experimental approaches below.

Highly susceptible mice can resolve primary infection with attenuated *Salmonella* strains and this requires a functioning immune system that can develop a $CD4^+$ Th1 cell response and IFN-γ production to activate infected macrophages (72–75). Similar experiments using attenuated bacterial strains show that mice lacking B cells resolve primary infection with similar kinetics to wildtype mice (76, 77), indicating that B-cell responses do not participate in primary clearance. $CD8^+$ T cells are generally not thought to contribute to primary clearance of attenuated *Salmonella*, based on studies using β2-microglobulin-deficient mice that lack class I-restricted $CD8^+$ T cells (73, 78). However, interpretation of these studies is complicated by the fact that β2-microglobulin-deficient mice lack CD1 and non-classical MHC molecules, and also have the potential to express free MHC class Ia molecules without β2-microglobulin (79, 80). Recent experiments with mice lacking classical MHC class Ia genes, perforin, or granzyme, show that $CD8^+$ T cells make a modest contribution to *Salmonella* clearance during the later stages of the primary response (81). Given the role of $CD4^+$ Th1 cells in production of IFN-γ and activation of macrophage microbicidal mechanism, it is not surprising that mice lacking Th1 cells due to a genetic deficiency in T-bet are unable to resolve primary infection with attenuated *Salmonella* (74). Similarly, mice lacking IL-12, IFN-γ, reactive oxygen species, or inducible nitric oxide, all have deficiencies in primary clearance of *Salmonella* (75, 82, 83). Overall, these data confirm a primary role for $CD4^+$ Th1 cells and an additional role for $CD8^+$ T cells in primary immunity to *Salmonella*. These activated $CD4^+$ and $CD8^+$ T cells produce the IFN-γ that is required for macrophage activation and bacterial killing. However, these findings are complicated by the fact that this infection model only examines immunity to highly attenuated bacteria. It seems possible, and even likely, that the dynamics of the adaptive immune response to highly virulent bacteria could differ substantially and the contribution of different cell populations could differ as a result.

To address this deficiency, many laboratories also examine protective immunity during secondary challenge of susceptible mice. Susceptible mice can resolve primary infection with attenuated bacteria over a period of 3–8 weeks, depending on the specific bacterial mutant, and following primary clearance are often immune to secondary challenge with virulent *Salmonella* (84). This infection model therefore allows examination of immunity to virulent bacteria in highly susceptible mouse strains. Since these mice resist secondary infection this model also allows for antibody-mediated depletion of cell populations or cytokines to assess the role of these in *Salmonella* immunity. These experiments have

confirmed the importance of both $CD4^+$ and $CD8^+$ T cells during protective immunity and demonstrated that IL-12, IFN-γ, TNF-α, and iNOS each play important roles in mediating bacterial killing (8, 9, 85, 86). It is surprising therefore that the adoptive transfer of spleen cells from immune to naïve mice is insufficient to transfer protective immunity (11). However, the transfer of spleen cells and serum from immune mice was shown to confer immunity to secondary challenge, although even this combined transfer provided lower levels of protection than intact immunized mice (11). The additional requirement for antibody in secondary protection suggests a fundamental difference in immunity against virulent and attenuated bacteria or in primary versus secondary clearance. Several studies using B cell-deficient mice have confirmed that B cells are essential for immunity to secondary challenge with virulent or attenuated *Salmonella* (76, 77). In some studies, the transfer of immune serum to immunized B-cell-deficient mice conferred the ability to resolve low dose secondary infection with virulent bacteria (76). This observation suggests that *Salmonella*-specific antibody can contribute to protective immunity during secondary infection as a supplement to *Salmonella*-specific $CD4^+$ and $CD8^+$ T cells. Indeed, this concept fits well with human studies showing that antibody is likely to be effective in immunity to typhoidal and non-typhoidal *Salmonella* (50, 52, 87). However, recent studies using B-cell-deficient mice or transgenic mice with peripheral B cells but no secreted antibody demonstrate that B-cell-mediated protection against secondary *Salmonella* infection can also be antibody independent (88). The protection in mice containing peripheral B cells correlated with robust Th1 cell responses to *Salmonella* antigens and was reduced in mice lacking B cells (88). Therefore, B cells make antibody dependent and antibody independent contributions to secondary protective immunity. Indeed, prior studies using B-cell-deficient mice have demonstrated a deficiency in Th1 cell responses in these mice during *Salmonella* infection (77, 89). Other studies have shown that mice with B cells lacking Myd88 develop lower Th1 and IgG2c responses during *Salmonella* infection due to an impairment in B-cell cytokine production that subsequently affects T-cell differentiation (90, 91). In conclusion, the numerous studies of vaccine-mediated protective immunity in susceptible strains of mice have confirmed a requirement for Th1 and CD8 T cells that was evident in studies with attenuated bacteria. In addition, vaccine-mediated protection to secondary infection also demonstrates an important requirement for B cells that can be evident in antibody-dependent and antibody-independent mechanisms.

Studies of vaccine-mediated protection are highly informative since they have the capacity to define the contribution of protective responses in the context of infection with virulent bacteria. However, it has to be remembered that all of these protective responses are initially induced by primary exposure to attenuated bacterial strains. Therefore, while these studies provide a great deal of mechanistic information about the immune response to a live vaccine strain of *Salmonella*, it is possible that the initial challenge with virulent bacteria that occurs in typhoid endemic areas is substantially different. As noted above, a response to primary infection can be studied using the resistant mouse model but is complicated by the fact that these mice rely primarily on antibody for bacterial clearance. One useful alternative therefore is to study host immunity to virulent bacteria in susceptible mice using antibiotic intervention to prevent fatal infection. Administration of Enrofloxacin to *Salmonella*-infected mice is highly effective at resolving infection with virulent strains and bacteria are

depleted from the spleen, liver, and bone marrow within 48–72 h of treatment (92). Interestingly, these antibiotic-cured mice develop a protective response that is evident upon secondary challenge but is ultimately insufficient to provide full protective immunity (92). Similar to vaccine-mediated protection, the limited protection in antibiotic-cured mice requires MHC class II-restricted T and IFN-γ, but also requires B cells (92). Thus, antibiotic-treated mice appear to generate a similar protective response to vaccinated mice but one that is insufficient to protect against secondary challenge. Delaying antibiotic intervention caused a corresponding increase in *Salmonella*-specific Th1 responses over a period of 7 days (93), suggesting that the Th1 response matures slowly over the first week of infection and is therefore susceptible to any interruption during this period. This model may be important for understanding why infected individuals can suffer from repeated bouts of typhoid in endemic areas, often after antibiotic intervention (19). Interestingly, a similar failure to acquire protective immunity after antibiotic treatment has been noted in *Chlamydia*-infected patients (94). Greater understanding of the antibiotic-treatment model may help define the maturation process of the Th1 response to intracellular bacterial infection.

While developing the antibiotic-treatment model using susceptible mice, it was found that an extremely long period of treatment was required to fully resolve primary infection (92). Although, 2–3 days of treatment eliminated bacteria from the liver, spleen, and bone marrow, up to 35 days of treatment was required to clear bacteria from mesenteric lymph nodes. Thus, if mice were treated with antibiotics for a week, bacteria recovered and proliferated within the MLN before spreading back to the spleen, liver, and bone marrow and causing fatal infection (95). This relapse of infection is due to the ability of *Salmonella* to respond to the intracellular environment by establishing a non-replicating population in some infected cells, a process that requires the expression of toxin-antitoxin modules (96). It is not yet clear why such a mechanism of dormancy would target bacteria to the MLN, but this lymphoid organ is also a site of persistent infection in resistant mice (97, 98). These resistant mice appear to have resolved primary infection but continue to shed bacteria in stools from this MLN reservoir (46, 97). Relapse of typhoid has also been described in endemic areas after antibiotic treatment (19, 99–101), suggesting that persistence and relapse in mice is an important phenomenon that requires greater study. It is not clear why the adaptive immune system fails to effectively combat relapsing primary infection, but again may be due to interruption of $CD4^+$ Th1 development by antibiotic treatment. Together, these studies of antibiotic treated mice further confirm the importance of $CD4^+$ Th1 cells in protective immunity to *Salmonella* and suggest that prolonged stimulation is required for these cells to develop.

Many studies in resistant mouse strains point to the effectiveness of antibody as a defense mechanism against *Salmonella*, despite the fact that these bacteria replicate intra-cellularly *in vivo*. Antibody-mediated defense is also clearly important in protective immunity to typhoid and non-typhoidal Salmonelosis in humans. Studies using highly susceptible mouse strains confirm an essential requirement for $CD4^+$ Th1 cells in resolving *Salmonella* infection, as has been noted in other intra-macrophage infections. However, even these studies demonstrate an important protective role for antibody-dependent and antibody-

independent B-cell responses. Thus, protective immunity to *Salmonella* infection is not simply a consequence of Th1 development but involves the activation of *Salmonella*-specific B-cell responses.

Antigen-specific reagents for studying *Salmonella*-specific immunity

The many studies outlined above provide an important overview of the host immune response to *Salmonella* infection, but a more detailed understanding of *Salmonella*-specific T and B-cell responses requires the ability to identify and monitor antigen-specific responses *in vivo*. This can be accomplished by generating recombinant *Salmonella* strains that then allow the use of established immunological tools or by developing new reagents specifically for use in the *Salmonella* infection model (12, 102).

TCR transgenic mice have a monoclonal population of peripheral T cells with defined antigen specificity and can be crossed to allelic backgrounds that allow direct identification of these T cells. Adoptive transfer of TCR transgenic T cells therefore allows direct tracking of a clonal T-cell population as it responds to antigen *in vivo* (103). This approach was initially applied to the *Salmonella* infection model by transferring OVA-specific $CD4^+$ T cells and infecting mice with *Salmonella* engineered to express OVA (104, 105). Later development of a *Salmonella*-specific TCR transgenic mouse called SM1 allowed direct visualization of flagellin-specific $CD4^+$ T cells to unmodified *Salmonella* strains (106, 107). Investigators have continued to use recombinant *Salmonella* strains expressing OVA or other antigens, combined with a variety of $CD4^+$ and $CD8^+$ TCR transgenic mice. Together, these different approaches allow direct visualization of the response of naïve *Salmonella*-specific $CD4^+$ and $CD8^+$ T-cell responses to a variety of different oral or systemic infections.

A second complementary approach has been to use MHC class I and II tetramers to monitor $CD4^+$ and $CD8^+$ T cells during *Salmonella* infection (13). Again, this approach can involve the use of recombinant *Salmonella* and established immunological reagents or the generation of new regents specific for the *Salmonella* model. However, while it is relatively straightforward to express model antigens in *Salmonella* and use established reagents, there are potential problems in interpreting these studies. First, the forced expression of any antigen in *Salmonella* can have adverse effects on bacterial growth and virulence and different promoter systems have been explored to ameliorate these detrimental effects *in vivo* (108). Second, it is not always clear whether the T-cell responses being visualized actually contribute to bacterial clearance. While this may seem a relatively minor issue, it has the potential to render many experiments as completely meaningless to *Salmonella* immunity. For example, in experiments using antigen-expressing *Salmonella* strains it is sometimes observed that prior immunization with this same antigen does not affect the clearance rate of the recombinant bacterial strain. Thus, although an antigen-specific T-cell effector population is activated, present, and can be directly visualized, it does not affect *Salmonella* clearance. If this is the case, one might argue that monitoring this antigen-specific population is something akin to visualizing T-cell responses to vaccination that just happens to occur in the context of a *Salmonella* infection. The danger in this approach is that this particular response may provide absolutely no direct information about *Salmonella*-

specific immunity at all. One way to avoid this criticism is to establish that prior immunization with a model antigen actually affects subsequent infection with a *Salmonella* strain expressing the same antigen, or alternatively, demonstrating that adoptive transfer of antigen-specific T cells affects bacterial clearance. A final concern with using recombinant *Salmonella* strains is the inherent inability of this approach to study the heterogeneity of the T-cell response to infection. It is often assumed that the T-cell response to a given pathogen will display a relatively uniform tempo and therefore studying one individual response provides a snapshot of the overall response to infection. However, given the complex regulation of bacterial antigen expression *in vivo*, this seems unlikely to be true and indeed has been challenged by recent studies in *Salmonella* infection models. Despite these potential concerns, our understanding of *Salmonella* immunity has benefited greatly from the use of antigen-specific reagents, both those designed specifically for this infection model and those that have been adapted from other systems. The results of some of these recent studies are discussed in more detail below.

Heterogeneity of T-cell response to different bacterial antigens

The first study to successfully characterize *Salmonella*-specific $CD4^+$ T-cell clones identified the target antigen of these T cells as an I-A^k epitope within the central hypervariable portion of bacterial flagellin encoded by the *FliC* gene (109). A subsequent study found an I-A^b epitope towards the conserved carboxy terminus of flagellin and this epitope sequence is the same in both *Salmonella* flagellin genes (FliC and FljB) (110). Subsequently, additional MHC class II epitopes were identified in the same protein and flagellin remains the most thoroughly defined target antigen in *Salmonella* infection (111, 112). Additional studies have shown that immunization with flagellin provides a modest degree of protective immunity to *Salmonella* infection, usually defined by modestly lower bacterial counts or a delay in time to death after infection (110, 113). Thus, flagellin is a well-defined target antigen of $CD4^+$ T cells during *Salmonella* infection and this response is likely to contribute modestly to protective immunity *in vivo*.

The T-cell receptor was cloned by PCR from a flagellin-specific T-cell clone, and the sequence was used to generate the SM1 TCR transgenic mouse (106, 110). After backcrossing this mouse to RAG-deficient and CD90.1 congenic backgrounds, the SM1 RAG-deficient CD90.1 mouse provides a useful source of T cells to visualize *Salmonella*-specific immunity *in vivo* (106). The early studies using this system focused on the initial stages of $CD4^+$ T-cell activation and demonstrated that the first activation of *Salmonella*-specific $CD4^+$ T cells occurs in the Peyer's patch and MLN within 6–12 h of oral infection (106, 114). This confirmed earlier studies that had identified these intestinal lymphoid tissues as the initial site of $CD4^+$ T-cell activation using OVA-specific reagents (105). Further studies using SM1 T cells demonstrated that early activation of *Salmonella*-specific T cells in the Peyer's patch required $CD11c^+$ dendritic cells and the chemokine receptor CCR6, expressed by PP DCs close to the epithelial layer (114). Thus, *Salmonella* flagellin-specific $CD4^+$ T cells are activated very early after oral infection and this process requires migration of $CCR6^+$ DCs within the infected lymphoid tissue. Although these findings fit with our understanding of the spread of *Salmonella* from PP, to MLNs, and eventually to systemic tissues (115), they are also somewhat at odds with a large body of *in vitro* work

demonstrating that *Salmonella* can inhibit DC presentation of antigen to naive $CD4^+$ T cells (116). Indeed, this disconnect between robust *in vivo* $CD4^+$ T-cell activation and inhibitory *in vitro* effects has not been fully resolved and will be discussed in more detail in the subsequent section. It should also be noted that this pattern of T-cell activation spreading from Peyer's patches to MLN and ultimately the spleen, is likely to be specific to oral infection in the absence of antibiotics. As noted above, if mice are pretreated with antibiotics, *Salmonella* can generate considerable intestinal inflammation and bacteria cross intestinal epithelial cells outside of Peyer's patches gain access to the underlying lamina propria in very large numbers (30). The presence of *Salmonella* in the *lamina propria* induces further inflammation and causes the activation and migration of *lamina propria* DC populations (117). However, in the absence of antibiotic-pretreatment, *Salmonella* infection of the epithelial layer is largely confined to the specialized M cells overlying intestinal lymphoid tissues such as Peyer's patches and solitary intestinal lymphoid tissues (SILTs) (106, 115, 118).

Although the SM1 adoptive transfer system targets a natural *Salmonella* epitope in C57BL/6 mice and therefore provides a unique ability to visualize *Salmonella*-specific immunity *in vivo*, the fact that these transgenic T cells recognize flagellin presents some difficulty in extrapolating to other *Salmonella* antigens. Immunologists have long known that bacterial flagellins are extremely potent immunogens and picogram quantities can induce immune responses in mice and rats (119–121). We now know that this potency is due to the expression of multiple innate receptors that can detect and respond to bacterial flagellins (122). The cell surface receptor TLR5 can bind flagellin and initiate a Myd88-dependent inflammatory response that involves cytokine and chemokine production and effective activation of DCs (123, 124). Thus, it is no surprise that flagellin can function as a potent adjuvant when it is co-administered with another protein (125). Furthermore, flagellin can be detected in the cytosol via NLRC4, which also initiates a distinct inflammatory pathway (122). These overlapping innate pathways have likely evolved to combat invasion by many different flagellated bacteria. In addition, flagellin is treated very differently from other antigens in that a subset of myeloid DCs in secondary lymphoid tissues possesses a unique capacity to efficiently process and present flagellin to $CD4^+$ T cells at very low antigen concentrations (126, 127). This potentiating effect is TLR5-dependent but strangely independent of the adapter protein Myd88 which mediates inflammatory activity downstream of TLR5 (126–128). Although the signaling pathway that mediates this effect has not been fully elucidated, it bears similarities to C-type lectin receptors that preferentially deliver bound antigens to the DC antigen-processing machinery (129).

Two other unusual features of the flagelin epitope recognized by SM1 T cells are also worthy of some consideration. First, the studies with SM1 T cells have demonstrated that flagellin-specific $CD4^+$ T cells are induced very rapidly but eventually this responses subsides (106, 107). This kinetic fits well with our understanding of *Salmonella* gene regulation since flagellin is heavily downregulated during the transition from extracellular to intracellular growth and therefore during the spread from the intestine to systemic tissues (130, 131). Indeed, SM1 T-cell activation can be evaded entirely if mice are challenged with a low dose of *Salmonella*, thus limiting the overall availability of flagellin *in vivo* (107).

Second, recent studies have been able to generate MHC class II tetramers specific for this same flagellin epitope, thus allowing direct visualization of the endogenous polyclonal response to flagellin (132). These studies have shown that the precursor frequency of the flagellin-specific $CD4^+$ T-cell population responding to the SM1 epitope is incredibly small (132, 133). In fact, there are around 20 flagellin-specific $CD4^+$ T cells in all the secondary lymphoid tissues of C57BL/6 mice, the smallest precursor frequency documented to date (134). While this may be no more than a peculiarity of the flagellin-specific repertoire, it accentuates the fact that artificially raising the precursor frequency by adoptive transfer has the potential to introduce unanticipated variables. While this is always true for TCR transgenic studies, it may be particularly true for studying flagellin-specific T-cell responses. Flagellin therefore generates an immediate inflammatory response, activates dendritic cells directly and indirectly, is preferentially processed and presented to CD4 T cells, is heavily downregulated by bacteria *in vivo*, and appears to have a uniquely low precursor frequency in C57BL/6 mice. Thus, while the development of SM1 T cells has allowed direct visualization of $CD4^+$ T-cell responses to a natural *Salmonella* epitope, it seems very unlikely that this response will be representative of other *Salmonella* antigens.

The challenge in dealing with this important issue is that very few other target antigens have been defined for *Salmonella*-specific T cells. Indeed, any attempt to generate $CD4^+$ T-cell clones from *Salmonella*-infected mice using bacterial lysates usually results in the generation of flagellin-specific T cells and very little else. This is probably due to a combination of the preferential processing and inflammatory activity of flagellin and could potentially be circumvented by the use of lysates from aflagellate bacteria or the use of TLR5-deficient spleen cells for *in vitro* stimulation. However, one recent paper has identified several new MHC class II epitopes of *Salmonella*-specific T cells using a combination of guesswork based on known targets of IgG and an unbiased bioinformatics approach (67). Interestingly, some of these new epitopes are found in SseI and SseJ, two effector proteins of a Type III secretion system (T3SS) encoded by *Salmonella* pathogenicity Island 2 (SPI2) (135). The collection of genes encoded by the SPI2 locus is expressed when *Salmonella* enter a macrophage and they are essential for survival. The proteins expressed by SPI2 form a needle complex that allows *Salmonella* to inject multiple effector proteins into the cytosol of the infected cell, thus modifying the phagolysosome to allow bacterial replication (135). Given the predicted cytosolic location of SseI and SseJ, it was surprising that these proteins would be presented in MHC class II and able to activate $CD4^+$ T cells. MHC class II tetramers were generated to visualize the $CD4^+$ T-cell response to both SseJ and flagellin, thus allowing the first comparative analysis of two different natural *Salmonella* target antigens simultaneously.

As might be expected from studies using SM1 T cells, the polyclonal $CD4^+$ T-cell response to flagellin was initiated rapidly, was primarily localized to the intestine, this response contracted despite the continued presence of *Salmonella in vivo* (67). In marked contrast, SseJ-specific $CD4^+$ T cells were activated a later, predominantly in systemic lymphoid tissues, and displayed no evidence of clonal contraction for several weeks (67). The starkly different kinetics of the $CD4^+$ T-cell response to these antigens was found to largely overlap with the kinetics of flagellin and SseJ expression by the bacteria *in vivo* (67). These data

suggest that flagellin is a dominant antigen target during the early response simply because it is highly expressed initially, while the SseJ-specific response is ongoing and continuous, reflecting it's antigen expressing *in vivo*. The kinetics of these two responses are also interesting from the standpoint of $CD4^+$ T-cell subset development since the flagellin-specific $CD4^+$ T-cell response contained a mixed Th1 and Th17 response that included IL-17 and IL-22 production, while the SseJ-specific T cells were largely Th1 cell. This difference correlates well with the different anatomical location of $CD4^+$ T-cell activation for these responses where the flagellin-specific Th17 cells preferentially develop in the intestine. While *Salmonella* is often thought of as an infection that generates Th1 cells, this is an oversimplification (16). While Th1 cells are critical for activation of infected macrophages to kill intracellular bacteria in systemic tissues, Th17 cells are probably essential for developing a neutrophil influx at the site of intestinal infection to engulf extracellular bacteria. The generation of IL-22-producing $CD4^+$ T cells should activate epithelial cells to produce antimicrobial peptides that are intended to combat free bacteria in the lumen of the intestine. It is interesting that two these different effector responses occur at different anatomical locations and also target unique antigens that are expressed by the bacteria at these different sites. Although additional antigen-specific reagents are required to confirm this divergent response to the same organism at different locations, together these data suggest a more complicated view of pathogen-specific T-cell responses where bacterial antigen regulation can be a major driver that determines the specificity and effector phenotype of the responding $CD4^+$ T-cell population. It will be interesting to determine whether the $CD4^+$ T-memory cell pool following bacterial clearance will retain this characteristic antigen targeting and effector phenotype.

Bacterial evasion of T and B-cell responses

As noted above, $CD4^+$ T cells, $CD8^+$ T cells, and B cells each contribute to protective immunity in the susceptible mouse model of *Salmonella* infection. Given the important role of these populations, it perhaps not surprising that *Salmonella* are thought to actively inhibit these responses (116). Inhibition of DC antigen presentation has been clearly demonstrated during the *in vitro* culture of *Salmonella*-specific T cells (116, 136). In some studies, *Salmonella* have been reported to induce apoptosis of infected dendritic cells (137, 138). In other reports, *Salmonella* did not directly affect DC viability but specifically blocked the presentation of peptides to responding $CD4^+$ and $CD8^+$ T cells (139–142). The effect of this inhibition is an overall reduction in T-cell clonal expansion and IL-2 production *in vitro*. This inhibitory effect required live bacteria and $CD4^+$ T cells can respond normally if bacteria are fixed or killed prior to culture (116). Interestingly, this inhibitory effect has been mapped to the SPI2 locus and $CD4^+$ T-cell activation is restored by using *Salmonella* strains with SPI2 mutations (140–142). The main problem in interpreting these *in vitro* studies is that an inhibitory effect of *Salmonella* on initial $CD4^+$ T-cell activation has not been detected *in vivo* (67, 106). In fact, as discussed above, a prominant feature of the SM1 T-cell response to infection is the rapidity of initial activation within intestinal lymphoid tissues (106). Robust and rapid $CD4^+$ T-cell responses to model antigens has also been described *in vivo* using *Salmonella* strains expressing recombinant proteins (104, 105, 143). Thus, it seems likely that there is no inhibitory effect of live bacteria on initial antigen presentation

in vivo. However, an alternative possibility is that the rapid $CD4^+$ T-cell response to infection that is detected *in vivo* is caused by DC presentation of secreted antigens or antigens recovered from dead bacteria and that active suppression of antigen presentation by live bacteria occurs later in the response. Either way, the *in vitro* culture conditions that are used to demonstrate *Salmonella* inhibition of naive T cells serve as a poor model of *Salmonella*-specific T-cell activation as it occurs *in vivo*.

Despite the inability to visualize any inhibition of *Salmonella*-specific $CD4^+$ T-cell priming *in vivo*, there is considerable evidence that *Salmonella* inhibit later stages of the $CD4^+$ T-cell response. If mice are immunized with soluble flagellin, responding SM1 T cells undergo a typical pattern of clonal expansion, subsequent contraction, and long-term survival of memory T cells (126, 144). In marked contrast, SM1 T cells responding to live *Salmonella* expand rapidly but do not survive beyond the clonal contraction phase (7). This failure to generate an expanded population of effector/memory SM1 T cells has been noted in both susceptible and resistant strains of mice and also affects the survival of bystander antigen-specific T cells responding to immunization (143, 145). Importantly, this inhibitory effect is not unique to flagellin-specific $CD4^+$ T cells and inhibition of OVA-specific, and Eα-specific TCR transgenic $CD4^+$ T cells has been detected after infection with *Salmonella*-OVA or *Salmonella*-Eα (143, 145). This loss of responding $CD4^+$ T cells is sometimes referred to ‘culling’, since it appears to be an active strategy that *Salmonella* use to evade antigen-specific $CD4^+$ T-cell responses *in vivo*. While the mechanism of this response remains undefined, again it is reported to require the expression of the SPI-2 locus (143, 145). Thus, despite the disparity between *Salmonella* inhibition of initial activation of naive $CD4^+$ T cells *in vitro* and *in vivo*, overall these studies point to an inhibitory effect of SPI-2 genes on $CD4^+$ T-cell responses.

A potentially confusing aspect of this *Salmonella* inhibition of $CD4^+$ T cells is that it is usually only apparent when studying the response of monoclonal TCR transgenic T cells *in vivo*. In contrast, when the endogenous polyclonal repertoire of responding *Salmonella*-specific T cells is visualized using MHC class II tetramers, this response shows evidence of expansion, contraction, development of effector cells, and eventually memory (67). It would be tempting to dismiss these inhibitory effects as simply an artifact of TCR transgenic adoptive transfer systems, especially since modulation of the naive T-cell precursor frequency can adversely affect T-cell memory in other systems (146, 147). However, careful titration of SM1 TCR transgenic T cells has demonstrated that these inhibitory effects of *Salmonella* can be detected across a wide range of precursor frequencies (143). Despite the apparent development of T cell effector responses in experiments studying endogenous $CD4^+$ T cells, it remains possible that this non-transgenic population experiences similar inhibitory effects to that reported for TCR transgenic T cells *in vivo*. Indeed, although a large population of IFN-γ producing T-bet^+ $CD4^+$ Th1 cells is detected as early as the first week of *Salmonella* infection (7), studies of mice lacking $CD4^+$ T cells or Th1 cells do not demonstrate a deficiency in bacterial clearance until 2 or 3 weeks after infection (73, 74). Thus, there is a notable disparity between the presence of Th1 cells and anti-bacterial effects due to the activity of these cells. Overall, these data would be consistent with a *Salmonella*-induced inhibitory effect on the endogenous $CD4^+$ T-cell response, just as has been

observed with TCR transgenic T cells. Since adoptively transferred TCR transgenic cells are non-renewable, it is possible that endogenous *Salmonella*-specific T-cell memory actually derives from new naive T cells that are activated later during infection. Indeed, although thymic output is significantly reduced during *Salmonella* infection, the thymus eventually recovers and may therefore provide the source of endogenous T cells that survive to seed the memory pool (148).

A direct inhibitory effect of *Salmonella* on T cells has also emerged from *in vitro* studies. This inhibitory effect is distinct from the mechanism discussed above since it can be observed in the absence of DCs and primarily appears to cause TCR downregulation, thus preventing T cells from receiving an activating stimulus (149, 150). Recent studies have provided greater detail about the mechanism of this inhibitory effect and have shown that it is due to expression of an L-Asparaginase II, which depletes asparagine from the culture medium (151). Since T cells require asparagine, this nutritional deficiency effectively blocks T-cell activation *in vitro. Salmonella* strains lacking L-Asparaginase II lack the ability to inhibit T cells *in vitro* and are attenuated *in vivo*. Together, these studies indicate that are multiple pathways that *Salmonella* uses to limit the proliferation or survival of effector $CD4^+$ T cells.

The absence of a *Salmonella* $CD8^+$ TCR transgenic mouse or MHC class I tetramer reagents has limited detailed analysis of *Salmonella*-specific $CD8^+$ T cells *in vivo*. However, some studies have used OVA-specific OT-I T cells, OVA tetramers, and *Salmonella*-OVA to examine $CD8^+$ T cells responding to *Salmonella* infection. Together these studies outline a surprisingly delayed kinetic of the $CD8^+$ T-cell response where initial activation and cell division is first detected only several weeks after infection (152). This delay in $CD8^+$ T-cell responses appeared to be due to the lack of early antigen presentation during the first weeks of infection (153). It is difficult to reconcile the very rapid response detected using $CD4^+$ T cells with the fact that the $CD8^+$ T-cell response appears to be significantly delayed. However, this pattern of lymphocyte activation is consistent with the kinetics of the B-cell response to *Salmonella* infection. Although isotype switched antibody responses are induced early after *Salmonella* infection, germinal center formation is delayed for several weeks (154). It is not yet clear whether a significant delay in $CD8^+$ and B-cell germinal center responses is a consequence of direct inhibition or whether these are simply secondary effects due to the absence of $CD4^+$ T-cell help. Together, they suggest that *Salmonella* have evolved effective strategies to hinder the development and function of adaptive immune responses. Uncovering the bacterial mechanisms that drive these inhibitory responses could be extremely informative for the design of improved live attenuated *Salmonella* vaccines with improved immunogenicity.

Role of non-cognate activation during effector T-cell responses

As noted above, many studies have demonstrated the important role of $CD4^+$ T cells in the resolution of *Salmonella* infection. Defects in Th1 cell development or the production or responsiveness to IFN-γ serve to increase susceptibility of infected mice (155). Furthermore, patients with inherited or acquired deficiencies in IFN-γ or IL-12 signaling can suffer from

Salmonella infections (24, 27). Thus, the activation and effector function of Th1 cells are critical for the resolution of *Salmonella* infection in humans and mice.

Initial activation of $CD4^+$ T cells requires two signals delivered by DC presentation of peptide/MHC complexes and costimulatory signals via B7 molecule ligation of CD28 (156). Thus, it is not surprising that mice lacking MHC class II molecules or CD28 are unable to resolve infection with *Salmonella* (73, 76). After expansion and differentiation of responding T-cell clones in lymphoid tissues, these effector T cells gain access to infected tissues where they can mediate bactericidal effects. Although the signals required for initial expansion and differentiation of $CD4^+$ T cells in lymphoid tissues are well defined, the signals required for eliciting IFN-γ response in infected tissue is less well understood. In mycobacterial infection, it has been shown that effector Th1 cells require recognition of peptide/MHC complex on the surface of an infected macrophage to kill bacteria (157). In contrast, during *Leishmania* infection, it has been shown that Th1 cells are activated to secrete IFN-γ locally and this cytokine gradient can activate infected cells irrespective of whether they are capable of presenting antigen or not (158). A third possibility is that activated T cells sense the presence of inflammatory cytokines in infected tissues and are activated to secrete cytokines in the absence of cognate ligand. This ability to respond to non-cognate signals essentially lowers the threshold for activation and may be advantageous during the immune response to a rapidly dividing pathogen (28). $CD8^+$ T cells can be induced to produce IFN-γ after stimulation by inflammatory cytokines such as IL-12 (IL-12) and IL-18 (159–161). Th1 cells can also respond to these same cytokines suggesting that non-cognate activation of Th1 cells could be important during *Salmonella* clearance (162, 163).

Studies examining the polyclonal response of $CD4^+$ T cells heat-killed *Salmonella* noted that a large proportion of $CD4^+$ T cells produced IFN-γ rapidly to this stimulus (7). This response was only detected in *Salmonella*-infected mice, suggesting that these $CD4^+$ T cells were responding to different *Salmonella* antigens via cognate stimulation. However, subsequent experiments demonstrated that this same response could be elicited by the injection of LPS and also required IL-18 (164). Thus, stimulation of the innate immune response caused activation of $CD4^+$ T cells in *Salmonella*-infected mice. In recent work, the mechanism of this non-cognate response has been examined in more detail (165). This study demonstrated that injection of *Salmonella*-infected mice with LPS, flagellin, or the TLR2 agonist PAM3CSK induced $CD4^+$ T cells to rapidly produce IFN-γ. By using a variety of mixed bone marrow chimeras, it was demonstrated that $CD4^+$ T cells did not respond directly to these TLR ligands but instead responded to IL-18 and IL-33 that were induced by innate stimulation (165). Surprisingly, this response was deficient in mice that lacked the major inflammasome components NLRC4 and NLRP3. Thus, synergy between inflammasome and TLR signals creates an inflammatory environment that induces CD4 Th1 cells to produce IFN-γ during bacterial infection. Importantly, mice containing T cells that lack this non-cognate pathway had a deficiency in primary clearance of *Salmonella* infection. Overall, these data indicate that non-cognate signals in infected tissues may be sufficient to activate Th1 cells during *Salmonella* infection. The ability of Th1 cells to

respond to non-cognate signals may have evolved to combat co-infections but this has not been demonstrated experimentally.

Conclusions

The *Salmonella* infection model has been widely used by immunologists attempting to understudy the innate and adaptive immune response to infection. Both the resistant and susceptible mouse model are attractive since they allow an examination of the protective contribution of $CD4^+$ T cells, $CD8^+$ T cells, and B cells in an experimental model that is easy to work with in the laboratory. Recent experiments have examined antigen-specific T-cell responses to *Salmonella* using recombinant bacterial strains with established immunological tools or new tools that have been developed for the *Salmonella* infection model. Together these experiments have provided a more detailed understanding of the adaptive immune response to *Salmonella* and a picture is emerging that is considerably more complicated that previously envisaged. Both the nature and location of antigen expression can regulate the simultaneous expansion of unique T-cell populations that are enriched in different anatomical compartments and have different functional activity. *Salmonella* appear to have an array of suppressive or inhibitory effects to affect responding T cells and B cells but the exact mechanism of these responses is largely undefined. The clearance of bacteria from host tissues appears to rely on a combination of cognate recognition of infected cells and non-cognate activation of previously expanded T cells. Although additional work is required to understand how these issues affect the development of protective immunity, each of these issues have the potential to improve vaccine development for typhoid and non-typhoidal Salmonellosis.

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