The Effects of Systemic and Mucosal Immunization on Liver Tissue-Resident Memory CD4 T Cells During Systemic *Salmonella enterica* Serovar Typhimurium Infection

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Dedication

This work is dedicated to my family for their unwavering love, support, and encouragement, to my friends whose motivation and kindness kept me inspired, and to the teachers, mentors, and lab mates who have nurtured my passion and understanding of science throughout the years.

To those who have left a lasting footprint in my life during my time as a graduate student – thank you for your constant encouragement as I navigate uncertainty and the unknown. You've motived me even when progress felt elusive, reminded me that failure is an integral part of discovery, that I do not have to face challenges alone, and that I have the power to define and refine my own success.

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Abstract

Salmonella enterica serovar Typhimurium is a significant health burden, affecting both humans and a wide range of companion animals. Infections can become systemic, leading to invasive non-typhoidal salmonellosis (iNTS) and can cause severe clinical outcomes that range from an asymptomatic carrier state to enteritis, endotoxemia, septicemia, and even death, with a conservative estimate of 200,000 deaths annually. Asymptomatic or latent carriers may shed salmonellae intermittently, increasing the reservoir of environmental *Salmonella* and can also develop clinical disease under stress, when immune system is compromised. Antibiotic resistance among *Salmonella* strains continues to rise, and most metabolic pathways have already been targeted or considered for drug inhibition, limiting the potential for new drug treatments. Furthermore, reinfections remain common, indicating incomplete protective immunity after an initial infection. Developing an effective vaccine against systemic *Salmonella* is critical to protecting both symptomatic and asymptomatic carriers and combating these infections. Advancing our understanding of the host immune response to *Salmonella* is essential for creating effective vaccine strategies.

A pool of CD4 memory T cells, particularly tissue-resident memory (T_{RM}) CD4 T cells, serves as a crucial first line of defense upon pathogen re-exposure. Therefore, an effective vaccine would incorporate the formation of CD4 T_{RM}, however, the most protective originating source of CD4 T_{RM} and what mechanism differentiating the protective CD4 T_{RM} populations are unknown. Previous research has shown CD4 T_{RM} in the liver, the main non-lymphoid tissue infected by systemic *Salmonella*, is required for complete protection against reinfection but the degree of protection has not been

compared with mucosal CD4 T_{RM}, the latter being of interest as *Salmonella* infections are acquired orally. While the infection begins in the small intestine, where bacteria adhere to and invade the mucosa, the majority of bacterial replication occurs in the lymphoid follicles, liver, and spleen.

Additionally, T cells have been observed to respond to non-cognate stimuli, enabling them to secrete cytokines in response to local innate stimuli without requiring the presence of a specific antigen. While this phenomenon is well-documented in CD8 T cells, where IL-12 and IL-18 signaling drive responses to viral infections, it remains unexplored in the context of CD4 T cells during bacterial infections. We hypothesize that non-cognate stimulation is a key factor differentiating the protective capabilities of mucosal and systemic CD4 T_{RM} in combating *Salmonella* infection.

To investigate this mechanism, we used an IL-18R floxed mouse model crossed with CD4-Cre to selectively eliminate IL-18R expression in CD4 T cells. The mice is immunized with a live vaccine strain (LVS) of *Salmonella*, allowing us to track immunization-specific CD4 T cells using tetramers. This model enables us to characterize mucosal and systemic CD4 T_{RM} populations and assess the impact of IL-18R deficiency on their respective functions. Furthermore, we performed adoptive transfers of isolated mucosal and systemic CD4 T_{RM} cells to evaluate the protective capacity of each population. This study demonstrated that adoptive transfer of hepatic T_{RMs} was sufficient to protect mice from challenge infection whereas T_{RMs} isolated from the intestinal lamina propria (LP) reduced bacterial loads but did not provide complete protection. The protective effects of both liver and LP T_{RMs} was dependent on expression of IL-18R and their ability to mount a non-cognate response in vivo. These findings highlight a critical

mechanism for enhanced T_{RM} protection, offering valuable insights to vaccine development and advancing our understanding of understudied areas in basic immunology.

Chapter 1

Introduction

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Introduction

Salmonella enterica is a gram-negative intracellular pathogenic bacteria that has long been a global health concern due to its impact on public health, particularly in low-income nations (1, 2). *Salmonella enterica* is a single bacterial species that comprises a diverse group of subspecies and serovars that cause a range of clinical disease in humans. Usually, the severity of these infections is highly dependent on the bacterial strain involved and the underlying susceptibility of the infected host. Together, *Salmonella* infections pose a growing challenge, particularly in endemic regions where inadequate sanitation and/or limited access to clean water facilitates human-to-human transmission. While *Salmonella enterica* serovars Typhi and Paratyphi cause systemic enteric fever (primarily in South and Southeast Asia), serovars such as Typhimurium and Enteritidis usually cause gastroenteritis in healthy individuals (2-4). However, certain strains of Typhimurium and Enteritidis can also cause invasive nontyphoidal Salmonellosis (iNTS), a common bloodstream infection in sub-Saharan Africa with a high fatality rate in young children (5, 6).

Clinical manifestations of Salmonellosis

In the United States (U.S.), a common cause of Salmonellosis is contamination of fresh food or produce from zoonotic sources, with individual outbreaks caused by different serovars (7). Symptoms usually begin around 6 to 48 hours after ingestion of contaminated food, an incubation period that is likely dependent on the dose of ingested bacteria. Headache and diarrhea are common, but eventually intestinal infection can cause nausea, vomiting, and severe abdominal pain (8, 9). *Salmonella* gastroenteritis is

usually self-limiting and resolves in 2-7 days, often without anti-microbial intervention. While the individual impact of these gastrointestinal infections may seem minor, large outbreaks linked to a contaminated food supply can have serious economic repercussions for food manufacturers (8, 10).

In low-income countries, the impact of Salmonellosis is more substantial than that observed in high-income nations (2, 11). In these geographic locations, *Salmonella* can cause life-threatening systemic infections due to serovars that primarily utilize human-to-human transmission (12). There are three main causes of fatal systemic Salmonellosis, (i) serovar Typhi, (ii) serovar Paratyphi A, and (iii) iNTS strains, although clinical disease caused by Typhi and Paratyphi serovars are identical and usually referred to collectively as enteric fever (or typhoid). According to the World Health Organization (WHO), *Salmonella enterica* serovars Typhi and Paratyphi A were responsible for 14.3 million cases of enteric fever worldwide in 2017, resulting in approximately 135,000 deaths (13, 14). While enteric fever is detected in many parts of Asia, Africa, and South America, the highest burden of disease is localized to South-East Asian countries (15). After an incubation period of 7 to 21 days, nonspecific symptoms commence in infected patients, including nausea, headache, constipation and myalgia, before systemic manifestations appear including fever, joint aches, and hepatosplenomegaly (2, 16). Interestingly, gastrointestinal symptoms are often not detected in these patients, despite *Salmonella* using an intestinal route of infection. If present at all, intestinal symptoms typically resolve before systemic fever commences (17, 18). The systemic nature of enteric fever is caused by widespread bacterial replication in the reticuloendothelial system of the spleen, liver, and bone marrow, an anatomical niche that some *Salmonella* serovars have evolved to

exploit. It is important to note that Typhi and Paratyphi do not infect animal species and have actually lost multiple genes that can promote intestinal replication (4). Thus, the pathogenesis of systemic infection is distinct from simple gastroenteritis caused by zoonotic transmission of non-typhoidal serovars. Due to an array of overlapping symptoms with many febrile infections, the impact of enteric fever is likely understated, especially in endemic regions where diagnostic microbiology laboratories are uncommon (11).

For every 10 cases of enteric fever caused by *Salmonella Typhi* infection, there are typically one or two cases caused by Paratyphi A or B (or rarely, Paratyphi C) (19, 20), although this rate is higher in certain geographical locations. The surface polysaccharide of Paratyphi A and B consists of linked terminal O repeating units and is exposed to the immune system, unlike serovar Typhi, which expresses a capsular Vi polysaccharide which covers surface polysaccharides (21, 22). The sugars comprising the core unit, and their linkages to one another, determine the overall structure of terminal O-polysaccharides of *Salmonella* and are an important criteria for classification (23, 24). Paratyphi A belongs to serogroup A, Paratyphi B is in serogroup B, and Typhi is placed in serogroup D, with all 3 serotypes sharing a common tri-saccharide backbone, but with different attached sugars (21, 22). Although Paratyphi A belongs to a completely different serogroup than Typhi, these organisms are closely related and cause similar disease. In contrast, only certain strains of Paratyphi B cause enteric fever, while the genome of Paratyphi C is more closely related to other *S. enterica* serovars than it is to Typhi (25).

Invasive non-typhoidal Salmonellosis (iNTS) is a fatal systemic disease, distinct from enteric fever, that causes 77,500 deaths per year. Unlike the geographical

distribution of enteric fever, the highest burden on iNTS disease is found in sub-Saharan Africa (26, 27). While this disease is caused by non-typhoidal serovars that typically induce gastroenteritis in the US, the bacterial strains causing iNTS in Africa are genetically distinct from strains responsible for food outbreaks (12, 28). These iNTS infections are primarily transmitted human-to-human and (like enteric fever) the primary symptoms of disease are systemic, rather than localized to the gastrointestinal tract (29). As with enteric fever, iNTS causes fever, joint aches and hepatosplenomegaly, accompanied by other nonspecific manifestations like nausea and headaches (5). Given the similar geographical distribution to typhoid, iNTS infection is also thought to be under-reported (11). Unlike enteric fever, severe disease caused by iNTS infections is associated with underlying immune susceptibility of the affected population (5). For example, iNTS infections are often linked to co-infection with malaria (30), perhaps due to the immune-suppressive effect of parasite infection (31, 32). Infections with iNTS also occur frequently in infants where they are an important cause of neonatal sepsis (5). A second spike of disease is observed in adults aged 25-40 years, the demographic containing the highest incidence of HIV infection (5). Other factors that contribute to increased iNTS infections include sickle cell anemia, malnutrition and, in rare cases, patients with underlying genetic deficiencies in IFN-γ or IL-12/23 signaling pathways (6). To summarize, the impact of Salmonellosis in high-income nations derives largely from zoonotic infections from contaminated food and primarily involves self-limiting gastrointestinal infection. In marked contrast, human-to-human transmission of genetically distinct *Salmonella* serovars occurs in low-income regions and these bacterial strains display a preference for replication in systemic tissues.

Current treatment, vaccines and vaccine development

Two major issues that increase the impact of global *Salmonella enterica* infections are growing antimicrobial resistance and the lack of effective vaccines for several serovars (33, 34). Indeed, these issues are related since an advantage of newer typhoid conjugate vaccines is their ability to prevent disease caused by strains with significant antimicrobial resistance (22). Typical treatment for systemic *Salmonella* infection still relies heavily on antimicrobial use and there is an increasing incidence of antibiotic resistance detected among clinical isolates (28). The primary antibiotics for treating enteric fever are fluoroquinolones, but growing resistance means that alternative antibiotics are often required, such as cephalosporins and azithromycin (33, 34). Unfortunately, *Salmonella* strains resistant to cephalosporins have also been reported and resistance to azithromycin has become common (2, 28). Due to the emergence of these multi-drug resistant strains, enteric fever is increasingly difficult and expensive to treat (14). While the development of new antibiotics is always a priority, the development of effective vaccines is the most likely route to a lasting reduction in fatalities associated with systemic Salmonellosis (22). A variety of different vaccine approaches have been explored over the years, yet most of these have narrowly targeted enteric fever caused by Typhi serovars.

Killed whole cell vaccines

The simplest vaccine for many gram-negative bacterial infections is immunization with a killed whole cell preparation generated by heat-treatment or fixation (22, 34). Indeed, parenterally administered inactivated whole-cell vaccines were widely used to provide

protection against enteric fever (35). As might be expected, adverse effects were commonly reported with this vaccine approach (9-34% of whole cell vaccine recipients). Thus, these vaccines are simple to develop, but are ultimately of limited utility in endemic regions (35). Currently, whole cell vaccines are not recommended for use in typhoid endemic areas due to these high rates of reactogenicity.

Live attenuated vaccines

Several live attenuated strains of *Salmonella* have been developed over the years, most with genetic lesions in essential metabolic pathways (36, 37). These attenuated strains are usually administered orally, display limited replication in systemic tissues, and can induce appropriate humoral and cell-mediated immune responses to bacterial antigens (17). In 1989, a live attenuated vaccine for typhoid, called Ty21a, was licensed in Europe, with licensing following shortly after in the U.S. (38). Ty21a is an orally administered vaccine derived from an attenuated Ty2 strain of *S.* Typhi that lost Vi Capsular Polysaccaride (ViCPS) expression (21). While this vaccine can be effective in endemic areas, it requires storage at 2 to 8°C, has a complex administration protocol, induces modest protection (45% at 1-3 years post-vaccination), and requires yearly boosting for travelers, or boosters every 3-5 years for patients in endemic areas (39). Additionally, Ty21a is only approved for children over 6 years old, despite infants often being adversely affected by this disease (6). Indeed, live vaccines can pose some danger to immune-compromised individuals, especially if they can be shed in feces or have the capacity to revert to virulence. Given the vulnerability of patient groups susceptible to

iNTS, it is not clear that a live attenuated vaccine is the most appropriate choice for use in endemic areas.

Capsule polysaccharide vaccines

The ViCPS vaccine was first licensed in the U.S. in 1994 and is still used for typhoid (22). Unfortunately, the protective efficacy of this vaccine is short-lived and declines rapidly after administration (40, 41). Additionally, ViCPS is only licensed for children over two years of age since (like most polysaccharide formulations) it has low effectiveness in infants (22). Mechanistically, polysaccharide immunization fails to engage CD4 T cell responses, meaning that polysaccharide-specific B cells can be activated, but germinal center formation, immunoglobulin class switching, and affinity maturation fails to take place (42). Thus, although ViCPS immunization can be effective for a short period, it does not provide the long-term protective immunity necessary to protect vulnerable populations living in endemic regions (34).

Polysaccharide conjugate vaccines

In 2017, the first typhoid conjugate vaccine (TCV), Vi-TT (Typbar), which had previously been used in India, was recommended for use by the World Health Organization (43, 44). This vaccine consists of *Salmonella enterica* serovar Typhi ViCPS directly conjugated to tetanus toxoid and has an efficacy of 82-85% in various studies (45-47). Other Vi-conjugate vaccines have since been introduced, including ViCPS conjugated to a nontoxic mutant of diphtheria toxin (Vi-CRM197) and a similar ViCPS-DT vaccine developed in South Korea (48, 49). Conjugating ViCPS to a protein antigen

(tetanus toxoid, diphtheria toxin receptor or CRM197) allows these vaccines to engage CD4 T follicular helper cells that encourage germinal center formation and long-lived memory B cell responses (42). However, this conjugate polysaccharide approach does not work for Paratyphi A or iNTS serovars since these serovars completely lack ViCPS expression (22). Thus, despite the improved efficacy of conjugate vaccines for typhoid, alternative approaches will be required to develop effective vaccines for Paratyphi and iNTS disease. In summary, effective vaccines for typhoid have now been developed but there are no vaccines available for disease caused by Paratyphi infection or iNTS. Since these bacteria cause over 50% of systemic disease, there is an urgent need to increase understanding of immunity to systemic Salmonellosis and develop additional vaccines.

Developing an improved understanding of *Salmonella* immunity

Mouse models to study systemic Salmonella infection

Animal models can allow a detailed mechanistic understanding of immunity to bacterial infections and an opportunity to assess candidate vaccines prior to human clinical trials (34, 50). The standard approach to studying immunity to systemic *Salmonella* infection uses an iNTS mouse model with serovar Typhimurium infection (51). Oral infection of C57BL/6 mice with *Salmonella* Typhimurium causes systemic disease with bacterial replication occurring in the spleen, liver, and bone marrow, thus somewhat resembling enteric fever caused by *Salmonell*a Typhi (52). There are two distinct experimental approaches to this systemic mouse model, depending on whether susceptible or resistant in-bred mouse strains are being used (4). In one approach,

susceptible mouse strains with a naturally-derived *Nramp1* mutation (C57BL/6 or BALB/c) are used to understand *Salmonella* pathogenesis and the basis of acquired immunity (53-55). *Nramp1*, also known as *Slc11a1*, is a gene that encodes a cation transporter that restricts nutrients such as iron and manganese from macrophage phagosomes, thereby conferring resistance to intramacrophage pathogens (56). Interestingly, recent work has challenged this basic understanding and suggests instead that Nramp1 might primarily function to restrict *Salmonella* growth in neutrophils (57). Though the exact mechanism remains unknown, the outcome of Nramp1 deficiency is that C57BL/6 mice are incredibly susceptible to *Salmonella* Typhimurium and succumb to infection within 5-10 days of inoculation (58). A major advantage of using this susceptible model is that these same strains can gain robust immunity to a secondary infection if immunized with a live vaccine strain of *Salmonella* (36). Thus, although these mice die quickly from primary infection with virulent strains, the susceptible model allows for detailed investigation of acquired resistance to systemic disease (52, 59).

The second systemic model makes use of Nramp1-resistant mouse strains where the administration of a high bacterial dose via intraperitoneal, intravenous, or oral infection routes is necessary for fatal infection (4). However, when a low dose challenge is used, Nramp1 functional mice experience milder infection that either fully resolves or develops further into a chronic infection with intermittent bacterial shedding (60). This complementary model is extremely useful for studying the basis of persistent *Salmonella* infection and how bacteria can maintain fecal shedding in the face of an active immune response (61). It also allows for detailed investigation of *Salmonella* biofilm development in the gallbladder, an issue directly relevant for understanding asymptomatic bacterial

shedding (62, 63). Resistant mice can also be fed a lithogenic diet to initiate cholesterol gallstones that support biofilm formation, resulting in enhanced fecal shedding (62). It is thought that the protective physical barrier created by biofilm formation allows *Salmonella* to resist host-mediated clearance (64). It is important to note that, irrespective of the mouse genotype used in each of the two models discussed above (susceptible and resistant), prior disruption of the gut microbiota is never an essential component of these models.

Intestinal inflammation initiated by *Salmonella* infection can be studied using a streptomycin-pretreatment mouse model. This model involves oral pretreatment of mice with streptomycin to perturb the normal commensal microbiota and eliminate or reduce colonization resistance (65). This innovative approach provides a reliable mouse model of colitis that has been very useful for understanding the protective role of the microbiota and how various pathogens initiate inflammatory disease (66). However, since this intestinal inflammation is largely driven by innate immune response, it is not directly relevant to vaccine-mediated immunity to *Salmonella* (65). Furthermore, as noted above, systemic human Salmonellosis often occurs in the absence of intestinal inflammation (18). Thus, our discussion of host immunity to *Salmonella* below will largely focus on findings using the C57BL/6 iNTS mouse model, the main workhorse model for understanding adaptive immunity to systemic *Salmonella* infection (34). In summary, a variety of different mouse models are used to understand *Salmonella* pathogenicity and immunity and each has advantages depending on the particular question being addressed.

Salmonella pathogenesis: route of infection

Salmonella enters and survives in the infected host by making use of Type-III Secretion Systems (T3SS) encoded by *Salmonella* Pathogenicity Islands (SPI)-1 and SPI-2 (17, 18). Exposure to an acidic environment within the stomach initially causes *Salmonella* to express SPI-1, encouraging invasion of the epithelial cells overlying Peyer's patches in the distal ileum (67). Specialized M cells within the follicle-associated epithelial layer are targeted for invasion by both SPI-1-dependent and SPI-independent mechanisms (68, 69). Once *Salmonella* cross the intestinal epithelium, they enter the sub-epithelial dome of the Peyer's patch which contains macrophages and dendritic cells that can be infected (70, 71). From this initial tissue location, bacteria rapidly spread to local mesenteric lymph nodes via lymphatic drainage and productively infect macrophages in this location (72). After being taken up by macrophages, *Salmonella* can sense acidification of the *Salmonella*-containing-vacuole (SCV) and in response express the T3SS/SPI-2-encoded needle complex (73). This second T3SS allows Salmonella to inject a variety of effector proteins into the phagocyte cytoplasm, thus modifying the SCV to fully support intracellular replication (68, 74). This whole process happens very rapidly and *Salmonella* can be detected in the Peyer's patches and mesenteric lymph nodes as early as 3 to 6 hours after oral infection (75). Once *Salmonella* has gained access to the mesenteric lymph nodes it can disseminate widely via efferent lymphatics and the blood stream, eventually entering the main systemic sites of replication, spleen, liver, and bone marrow (76). It should be noted that although *Salmonella* enters the host directly via the intestinal epithelium in the mouse model, there is usually very limited bacterial replication within the lamina propria itself and systemic tissues become the major niche for bacterial

growth. Thus, similar to human infection, bacteria primarily replicate in systemic tissues, rather than mucosal tissues.

Routes of infection in Salmonella mouse models

In most experimental studies of systemic *Salmonella* infection, inbred mice are infected systemically using a direct intraperitoneal or intravenous route of administration. If the oral route is used, it often involves administration of bacteria directly into the stomach using a blunt end gavage needle (77). Performing oral gavage can sometimes damage the esophageal lining, raising concerns about whether bacteria actually access systemic tissues via an intestinal route or whether it occurs directly via abrasions. Furthermore, the degree of physical manipulation required for animal restraint may cause stress that influences the subsequent immune response. These concerns led our laboratory to examine whether a more natural route of infection could be developed that mimics foodborne or contaminated water exposure. Inoculated food chow fragments have been used to study immunity to *Listeria* and *Salmonella* infection (78, 79). Similarly, our approach was to infect mice using contaminated water which leads to systemic bacterial loads very similar to intragastric gavage infections (80). Thus, food or water bottle contamination provides straightforward means of natural infection in this mouse model.

Innate immune responses to *Salmonella* infection

During any infection, the capacity to quickly recognize an invading pathogen is the first step towards initiating an appropriate inflammatory response. Since *Salmonella* is typically transmitted via the fecal-oral route, responsibility for initial recognition of the

bacteria is found in the intestine. Sensing of bacterial invasion by intestinal epithelial cells rapidly causes recruitment of local phagocytic cells to the Peyer's patch (59, 81). A variety of Pathogen-Associated Molecular Patterns (PAMPs) are recognized during this early phase of infection, including lipopolysaccharide (LPS) lipid A, flagellin, bacterial amyloid fibers, and CpG-rich repetitive elements in *Salmonella* DNA (81-83). These common bacterial elements are recognized by Toll-like receptors (TLRs) expressed by innate immune cells, or by intestinal epithelial cells themselves (83-85). Upon ligand binding, TLRs initiate rapid downstream signaling cascades that culminate in the secretion of chemokines and cytokines (81). These inflammatory mediators increase the bactericidal activity of local tissue macrophages, induce maturation and migration of monocytes and dendritic cells, and alter the local vasculature to accelerate influx of circulating lymphocytes (81). This rapid response allows some degree of initial control of bacterial replication, while more sophisticated adaptive responses are developing in the local draining mesenteric lymph nodes (MLNs).

TLRs and NLRs

TLR2 and TLR4 are particularly crucial for the initial inflammatory response to *Salmonella* infection, since TLR2-, TLR4-, and TLR2/4-deficient mice display increased bacterial burdens in the MLNs after oral infection (73, 86). TLR5 induces a similar inflammatory response to *Salmonella* flagellin and this innate response can also enhance presentation of flagellin epitopes on MHC class II molecules to initiate a flagellin-specific CD4 T cell response (72, 87). It is not completely clear whether this early flagellin-specific CD4 T cell response is protective, but in some circumstances flagellin-specific CD4 T cells

can provide limited protection (88, 89). An alternative interpretation is that this strong early T cell response favors the bacteria since it serves to focus adaptive immunity onto flagellin while the bacteria simultaneously downregulate expression of this protein (90). Other reports show that TLR5 ligation can encourage bacterial dissemination from the intestine (91), reinforcing the idea that this process might be detrimental for the host.

Cytosolic Nod-like receptors (NLRs), namely NLRP3 and NLRC4, are also critical for the induction of inflammatory responses to *Salmonella* infection since these proteins sense cytoplasmic bacterial products and induce an inflammasome response that leads to the release of proinflammatory cytokines IL-18 and IL-1β (92, 93). NLR-dependent cytosolic detection allows host cells to discriminate between commensal bacteria and pathogenic bacteria like *Salmonella* that are able to inject products into the cytosol, one "pattern" of pathogenesis (94). Mice deficient in these NLRs display increased susceptibility to *Salmonella* infection, similar to mice deficient in caspase-1 or IL-18 (93, 95, 96). When mice lack caspase-1 expression but are supplemented with IL-18, this susceptibility is reversed (95), demonstrating that IL-18 is the ultimate goal of this inflammasome cascade. This innate circuit also becomes important to the regulation of subsequent adaptive immune responses, since production of IL-18 by innate immune cells dramatically enhances the capacity of *Salmonella*-specific Th1 cells to secrete IFN-γ (97, 98). While these early innate responses serve to restrain bacterial growth, ultimately the host requires adaptive immunity to resolve infection with *Salmonella*.

Adaptive immune responses to *Salmonella* infection

As noted above, *Salmonella*-specific CD4 T cells are activated within the first hours of oral infection (72, 99). However, this low frequency naïve population needs to expand and gain effector capability over several days before it can impact bacterial replication (100). CD4 T cell production of IFN-γ is first detected 3 days after infection, peaks around 7-14 days, and then eventually reaches a plateau that coincides with reduced bacterial growth (51). At the peak of this response, *Salmonella*-specific CD4 T cells account for 50% of all CD4 T cells within lymphoid tissues, with the vast majority expressing T-bet and secreting IFN-γ (59). These expanded T-bet$^+$ Th1 cells gain the capacity to migrate to nonlymphoid tissues such as the liver, where they eventually control bacterial replication via IFN-γ production (101). In marked contrast, T-bet-deficient mice display CD4 T cells with low IFN-γ and these mice eventually succumb to infection similarly to IFNγR-deficient mice (102-104), thus demonstrating the importance of Th1 development to *Salmonella* immunity. Although IFN-γ is also produced by innate cells to form iNOS$^+$ granulomas around bacteria, absence of Th1 CD4 T cells prevents mice from resolving infection (104). Other work has suggested a protective role for Th17 cells during *Salmonella* infection (105). As noted above, *Salmonella*-specific Th17 cells are detected early during mucosal infection and both IL-17 and IL-22 are produced in the intestinal mucosa at these early timepoints (106). IL-17A-deficient mice display a modest increase in bacterial dissemination after *Salmonella* infection, supporting a model where Th17 cells contribute to the mucosal barrier (107). In summary, CD4 Th1 cells are essential for the clearance of primary *Salmonella* infection, while Th17 cells can assist by hindering bacterial dissemination from the initial intestinal infection (Figure 1).

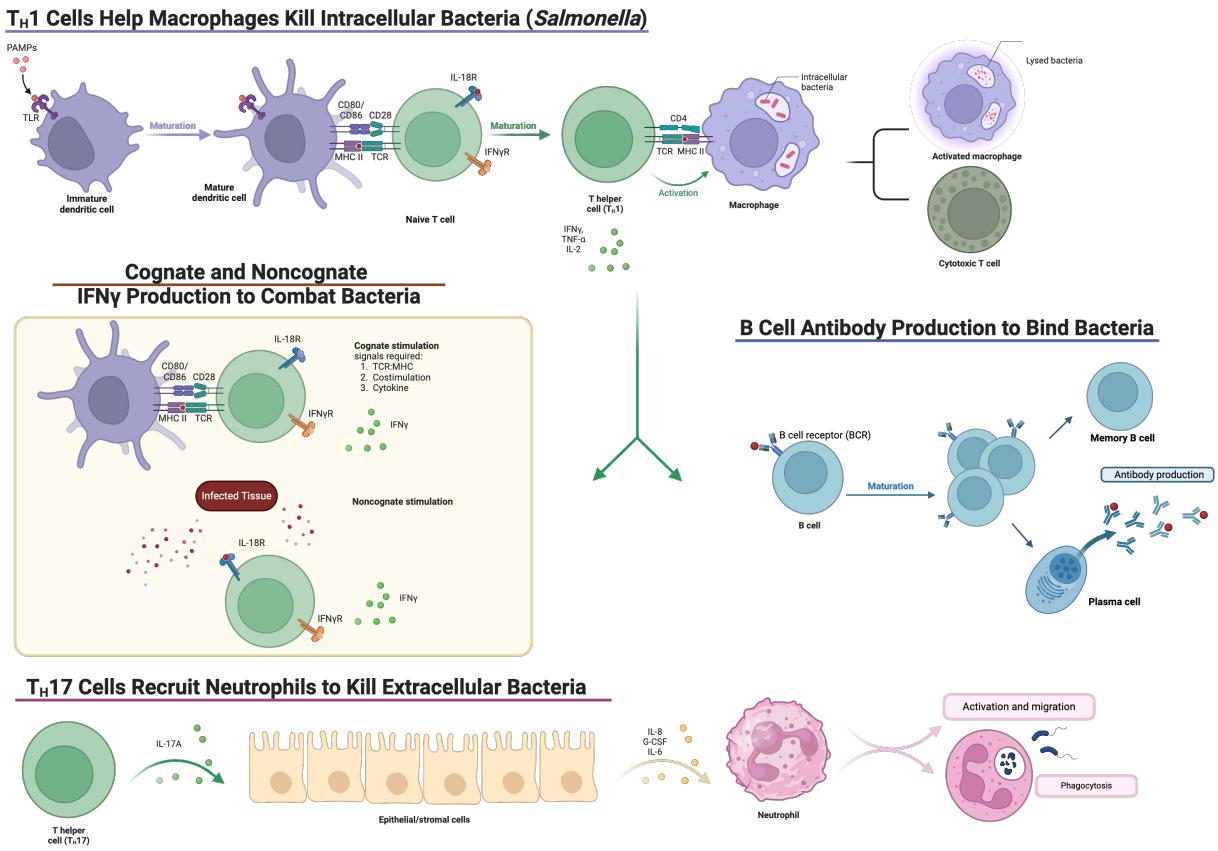

Figure 1. **Adaptive immune responses to combat *Salmonella* infection.** After activation of naïve T cells by antigen presenting cells, the naïve T cells will then differentiate into Th1 cells, further activating macrophages for intracellular killing. Additionally, with T cell help, T cells help drive B cell development, resulting in antibody maturation and proliferation, production and differentiation of plasma cells, and generation of memory B cells. Th1 cells can also be activated via cognate (MHC/peptide) stimuli or noncognate (such as IL-18) stimuli to produce IFN-γ to aid in resolution of infection. Furthermore, Th17 cells can recruit neutrophils to hinder bacterial dissemination.

Other adaptive immune cells

There is evidence that other adaptive immune populations play a supportive role to CD4 T cells during the clearance of *Salmonella* infection (108). For example, mice lacking MHC class-I-restricted CD8 T cells display a modest increase in bacterial burden during the late stages of primary infection (109), whereas mice deficient in B cells or NKT cells do not show any deficiency during primary infection (110-112). Some studies show that IFN-γ production by CD8 T cells prevent CD4 T cell-deficient mice from succumbing to *Salmonella* infection, supporting an additive protective role for CD8 T cells (113). While B cells are not required to resolve primary *Salmonella* infection, they play an essential role in secondary protection (110, 111). Strangely, this protective effect appears to be antibody-independent since mice resist secondary infection even if B cells are deficient in antibody secretion or isotype switching (114). Together, these studies support a central role for CD4 Th1 cells and a poorly defined supplementary contribution from CD8 T cells and B cells, especially during secondary infection (Figure 1).

Noncognate T cell stimulation

During *Salmonella* infection, expanded *Salmonella*-specific CD4 Th1 cells secrete IFN-g when they encounter infected macrophages in tissues (51). Although this Th1 cell activation can occur via TCR ligation, this effector response still occurs in the absence of cognate TCR stimulation (97). A similar phenomenon occurs during virus-specific CD8 T cell activation and involves responsiveness to local inflammatory cytokines such as IL-12 and IL-18, although many cytokines participate (115, 116). During bacterial infections, inflammatory cytokines such as IL-1β and IL-18 are often produced in infected tissues

due to TLR and NLR host recognition of PAMPs (93). This inflammatory environment allows effector CD4 Th1 cells to sense *Salmonella*-infected tissues and produce IFN-γ in a noncognate manner (97) (Figure 1). This innate capacity of adaptive Th1 cells is likely to be important because *Salmonella* can effectively downregulate MHC class-II expression on the surface of macrophages (117), thus inhibiting the potential for cognate interaction with peptide/MHC complexes. CD4 T cells can easily circumvent this bacterial evasion strategy by detecting local inflammatory mediators such as IL-18 and TL1a (97, 98). Additionally, when time may be a limiting factor, being able to mount a response without waiting for cognate APC:T cell interaction is beneficial. Mice with CD4 T cells unable to respond to IL-18 or TL1a show increased susceptibility to *Salmonella* (97, 98), demonstrating the importance of this activation mechanism during active infection. While the ability of *Salmonella* to downregulate MHC class-II seems to affect CD4 effector T cell responses, it clearly does not impact initial priming of a strong CD4 T cell response in the first few days of infection (99, 118, 119).

Memory T cells

The establishment of immunological memory relies upon the maintenance of expanded memory lymphocytes following bacterial clearance (120). Antigen-primed naïve CD4 T cells differentiate into effector or memory cells, characterized by their ability to mount a swift response upon re-exposure to antigen (121). After the resolution of infection, approximately 90% of effector T cells undergo apoptosis, resulting in the eventual emergence of memory T cells that possess the ability to self-renew and endure in the absence of peptide/MHC class-II ligand stimulus (122). These memory T cells

provide an immediate effector response to a previously encountered pathogen or as a consequence of vaccination. As noted above, CD4 Th1 cells are central for orchestrating *Salmonella* clearance during primary infection and also play the major role in protection against secondary infection (102). Thus an effective *Salmonella* vaccine should appropriately stimulate CD4 Th1 cells that elicit this same effector response.

Memory cells are heterogenous and were initially subdivided into two populations, T effector memory (T_{EM}) cells, and T central memory (T_{CM}) cells (123). Both of these memory populations are detected in blood, but express distinct surface receptors that facilitate their migration to different body tissues. T_{EM} cells display immediate effector function and survey peripheral nonlymphoid tissues where they are reactivated if they encounter antigen (124). T_{CM} cells lack effector functions, but express surface CD62L and CCR7 which allows them to migrate between blood and lymph nodes (123, 125). In recent years, a third subset of memory T cells has been identified within barrier tissues, but absent from blood (126). These Tissue-resident memory (T_{RM}) T cells are retained within tissues and appear to be situated in anatomical locations that facilitate a rapid response to re-infection. Like T_{EM}, T_{RM} display immediate effector function, enabling them to rapidly activate local innate and adaptive defenses during secondary challenge (127). Additionally, if memory T cells can be innately stimulated by responding to local inflammation, then this will be advantageous for pathogen clearance. Numerous studies have shown an essential role for T_{RM} T cells in providing protection against certain pathogens, suggesting that vaccines for these pathogens could be improved if this population can be elicited in vivo (128, 129). These T_{RMS} have also been detected in a wide variety of human tissues, supporting the idea that the heterogeneity of memory

subsets is a feature of human immune responses to infection (130). Thus, induction of T_{RMS} could potentially be the missing link for developing effective vaccines against intracellular pathogens like *Salmonella* since they require robust CD4 T cell effector responses in tissues.

After initial activation, T_{RMS} often retain surface expression of CD69 which inhibits the sphingosine-1-phosphate (S1P) receptor, a molecule that regulates migration of immune cells towards efferent lymphatics and lymph nodes (131). *Salmonella*-specific $CD69^+$ memory CD4 T cells in the liver have high expression of P2RX7, CD11a, CXCR3, and CD101, indicative of a T_{RM} phenotype (132, 133). Some memory CD8 T_{RMS} in barrier tissues express the integrin CD103 that also facilitates tissue retention, allowing tethering to skin or mucosal epithelial cells via interactions with E-cadherin, however, CD4 T_{RMS} rarely express this molecule (134). A growing catalogue of T_{RM} markers is reported, many of which are tissue-specific, suggesting that the local microenvironment instructs final T_{RM} differentiation (130). Beyond phenotypic and transcriptional profiling, functional definition of the T_{RM} contribution to pathogen immunity can be evaluated via parabiotic surgery or adoptive transfer (130). When this approach was used to examine T cell-mediated immunity to *Salmonella*, both circulating (T_{EM}/ T_{CM}) and resident (T_{RM}) CD4 T cells were shown to be necessary for complete protective immunity (89, 135).

Location of protective CD4 T_{RM}

CD4 T_{RMS} in mice have been found in numerous tissues including the lung, female reproductive tract, small intestine, large intestine, and liver, among others (126, 136, 137). As mentioned above, although *Salmonella* is transmitted via the fecal-oral route, few

bacteria are detected within the intestinal lamina propria, with instead the majority of replication occurring in the liver, spleen, and bone marrow. Of these sites, the liver would appear to be the most likely location for *Salmonella*-specific CD4 T_{RMS} to protect against a secondary infection. Indeed, examination of mice immunized with a live vaccine strain revealed that many *Salmonella*-specific hepatic CD4 T cells specific express a T_{RM} phenotype and that adoptive transfer of this population alone confers protection against *Salmonella* challenge (132, 135, 138). Thus, these recent data point to an essential role for hepatic T_{RM} Th1 cells in protection against *Salmonella* infection and help explain why adoptive transfer of splenic CD4 T cells from immune mice (containing low numbers of T_{RM} T cells) fails to provide the CD4 T cell-mediated protection observed in vaccinated mice. It is very likely that some *Salmonella*-specific T_{RM} can also develop within the intestinal lamina propria, but the role of these cells in protection against challenge is questionable since this organ is not a major site of bacterial replication. Overall, these new studies suggest that targeting T_{RM} formation in the liver, rather than mucosal sites, may be the best way to provide protection against systemic Salmonellosis.

The value of mucosal versus systemic immunity

As noted above, there is new interest in hepatic *Salmonella*-specific T_{RMS} because these cells appear to be necessary and sufficient for protection. Indeed, the overall contribution of traditional mucosal immune responses during *Salmonella* infection is debatable. This makes some sense when considering that the major anatomical location of bacterial growth is within systemic tissues rather than the intestine (17). The exact contribution of mucosal versus systemic immunity will be important to unravel because it

has obvious implications for whether mucosal or parenteral vaccination is required to protect against systemic *Salmonella* infections caused by Paratyphi or NTS strains. One study has examined a mouse model with reduced mucosal dendritic cells and reported that this deficiency had minimal impact on *Salmonella* infection in vivo, further supporting the importance of systemic over mucosal immunity (139). Additionally, the presence of a memory T cell population responding to local, innate stimuli is beneficial in most situations, but could also instigate harmful inflammatory response, especially in mucosal tissues as it is highly sensitive (140). In summary, while the assumption that mucosal immune responses are critical for protection is easy to understand, recent data would suggest that protection can be uncoupled from mucosal immunity and stronger priming of systemic responses is essential.

Conclusions

Systemic *Salmonella* infections are caused by a variety of serovars and remain challenging to deal with despite the availability of antimicrobials and an effective vaccine for typhoid. In particular, the development of prophylactic measures for Paratyphi and NTS infections needs urgent attention. Mouse models of systemic disease have been useful in uncovering the nature of lymphocyte defenses against reinfection. What we have learned from recent studies is that mucosal immune responses are not necessarily a critical variable in providing solid protection against Salmonellosis. Instead, adequate priming of systemic immunity appears to be the best defense against bacterial replication in tissues. In particular, a major requirement for hepatic T_{RMS} has been uncovered that offers new opportunities to consider the development of effective parenteral vaccines.

The development of sub-unit vaccines with adjuvants that can encourage T_{RM} formation in the liver is likely to be a useful approach. However, there are many outstanding questions that remain to be answered. Do *Salmonella*-specific hepatic T_{RMS} recognize a different repertoire of bacterial antigens that circumvent T cell and antibody responses? Do these T_{RMS} need to develop the capacity for non-cognate T cell activation in order to resolve infection with a bacteria that can suppress MHC class-II expression? Are there adjuvants that can initiate CD4 T cell expansion in the draining lymph nodes and also encourage T_{RM} formation in the liver? Could a new generation of Salmonella vaccines induce T cell responses that would provide overlapping protection against systemic disease caused by Typhi, Paratyphi, and NTS strains? Answering these important questions would not only provide a roadmap for *Salmonella* vaccination but may also assist in the generation of new vaccine approaches for other bacterial infections.

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Chapter 2

A deeper look into mucosal and systemic protection against mucosal *Salmonella* infection

Adapted from submission of paper:

Parenteral vaccination induces liver Tissue Resident Memory CD4 T cells and protects against mucosal *Salmonella* infection

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Introduction

Salmonella is a common contaminant of food and fresh produce, frequently linked to outbreaks of self-limiting gastroenteritis, often highlighted in U.S. media reports (1–3). However, globally, *Salmonella* poses a greater threat due to certain serovars' ability to cause severe systemic diseases. These infections predominantly affect low-income regions, where poor sanitation and limited access to clean water facilitate human-to-human transmission. *Salmonella enterica* Typhi and *Salmonella enterica* Paratyphi are human-specific serovars responsible for enteric (or typhoid) fever, a systemic illness with similar clinical manifestations (4, 5). Recent data indicate that enteric fever impacts 11–21 million individuals annually and results in 148,000–161,000 deaths worldwide (6, 7). Although Typhi and Paratyphi are transmitted via the fecal-oral route, the disease primarily involves systemic rather than intestinal symptoms (8). This systemic nature stems from the bacteria's affinity for the reticuloendothelial system, including the spleen, liver, and bone marrow (9). Similarly, a group of Non-Typhoidal *Salmonella* (NTS) serovars has evolved to cause invasive infections, particularly in young children and immunocompromised individuals in Africa and parts of Asia. This condition, known as invasive NTS (iNTS), represents a serious public health concern (10, 11). Recent advances, such as typhoid conjugate vaccines, have demonstrated high efficacy in reducing enteric fever in endemic areas by generating robust memory B cell responses to the *Salmonella* Vi capsule polysaccharide (CPS) (7, 12). Unfortunately, strains like Paratyphi and iNTS, which account for 50% of *Salmonella*-related deaths and 83% of fatalities in children under five, lack ViCPS expression, rendering these vaccines

ineffective (13). Developing new vaccines to address these strains requires a deeper understanding of *Salmonella* immunity.

For many years, *Salmonella* vaccine research has primarily focused on developing improved live attenuated vaccine strains (LVS) (14). The use of LVS vaccines is based on the reasonable assumption that mucosal immunity plays a crucial role in protecting against oral *Salmonella* infections. Numerous studies have explored *Salmonella*-specific antibody and T cell responses in the intestines of LVS-immunized mice and humans (15, 16). In mouse models, CD4 Th1 cells have been shown to be vital for protective immunity, as the secretion of IFN-γ is necessary to control bacterial replication within macrophages (17–19). However, early attempts to transfer lymphocyte-mediated protection from LVS-immunized mice using splenic Th1 cells were largely unsuccessful (9, 20). One hypothesis to explain this failure is the essential role of Tissue Resident Memory (T_{RM}) CD4 T cells in *Salmonella* immunity, with the spleen lacking sufficient T_{RMS} (21). This hypothesis has been confirmed, as the transfer of liver T_{RMS} from LVS-immunized mice conferred robust immunity, whereas circulating memory T cells did not (22–24). These findings have significant implications for *Salmonella* vaccine development, highlighting the need for vaccines that can generate liver T_{RMS} to provide effective immunity (16).

Given the critical role of *Salmonella*-specific liver T_{RMS} in providing protection, understanding how these memory T cells are generated following LVS immunization or infection is essential. Recent findings indicate that inflammatory cytokines expressed in the liver promote the development of *Salmonella*-specific T_{RMS} (25), suggesting that local tissue signals are necessary for their final maturation. Additionally, the expression of T-

bet and CD18 within T cells is crucial for Th1 T_{RM} precursors to develop into a liver-resident population (26). Considering that typical *Salmonella* infections or LVS administration occur via the oral route, it is also important to determine whether T_{RMS} in the intestinal lamina propria (LP) possess comparable protective capabilities. Moreover, given the established importance of liver T_{RMS}, it raises the question of whether immunity at the intestinal mucosa is even necessary. In this study, we examine the immune response to LVS immunization via oral and parenteral routes, finding that systemic priming effectively protects against mucosal *Salmonella* infection.

Results

Mucosal or systemic immunization can protect against oral or systemic infection

Although *Salmonella* is primarily a mucosal pathogen, the relative roles of mucosal and systemic lymphocytes in host protection remain unclear (29). To address this, we immunized susceptible C57BL/6 mice either orally or intravenously (IV) with an AroA-deficient live vaccine strain (LVS) of *Salmonella*. As expected (30), oral immunization protected mice from a subsequent oral challenge with the virulent SL1344 strain, which is typically fatal in naïve mice (Fig. 2.1A) and is not dose-dependent (Sup.Fig.2.1). Furthermore, oral immunization effectively protected most mice against IV infection (Fig. 2.1A), indicating that mucosal immunization induces robust systemic immunity. Next, we evaluated the protective effect of systemic immunization with LVS-*Salmonella*. Consistent with expectations, IV immunization provided strong protection against subsequent systemic (IV) infection with SL1344 (Fig. 2.1B). Surprisingly, IV immunization also effectively protected mice from oral challenge with *Salmonella*, demonstrating that parenteral immunization can confer protection against mucosal infection. To investigate this further, we quantified bacterial burdens in the spleen and liver. IV immunization significantly reduced bacterial loads, regardless of whether the challenge was delivered via a systemic (Fig. 2.1C and D) or oral route (Fig. 2.1E and F). These findings underscore that parenteral immunization offers robust protection against mucosal *Salmonella* infection.

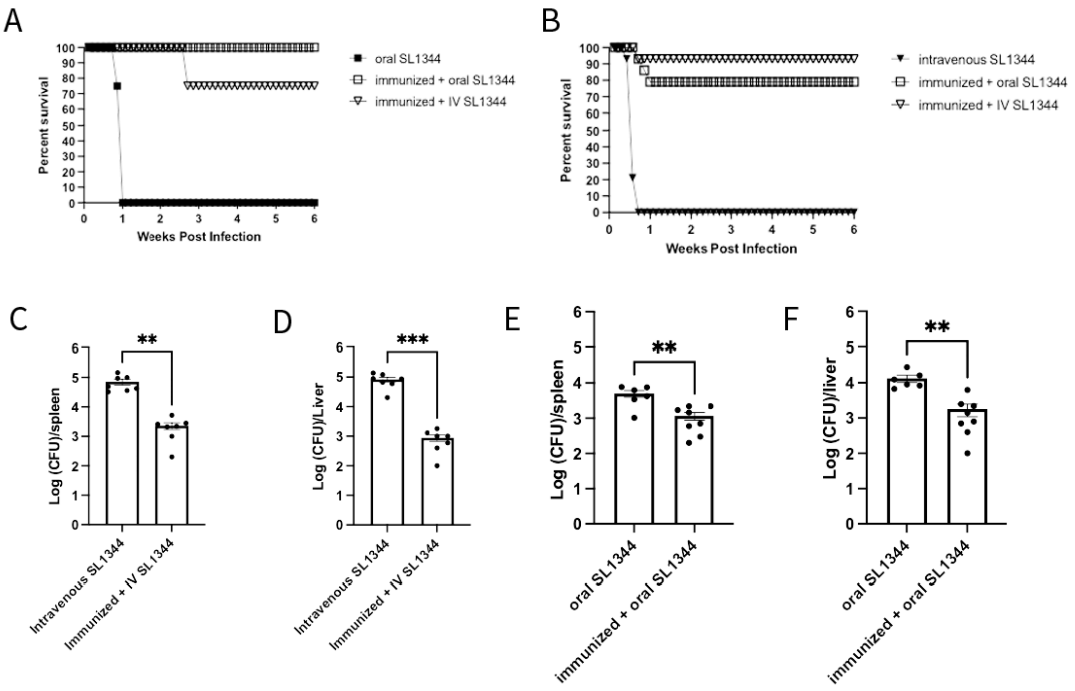
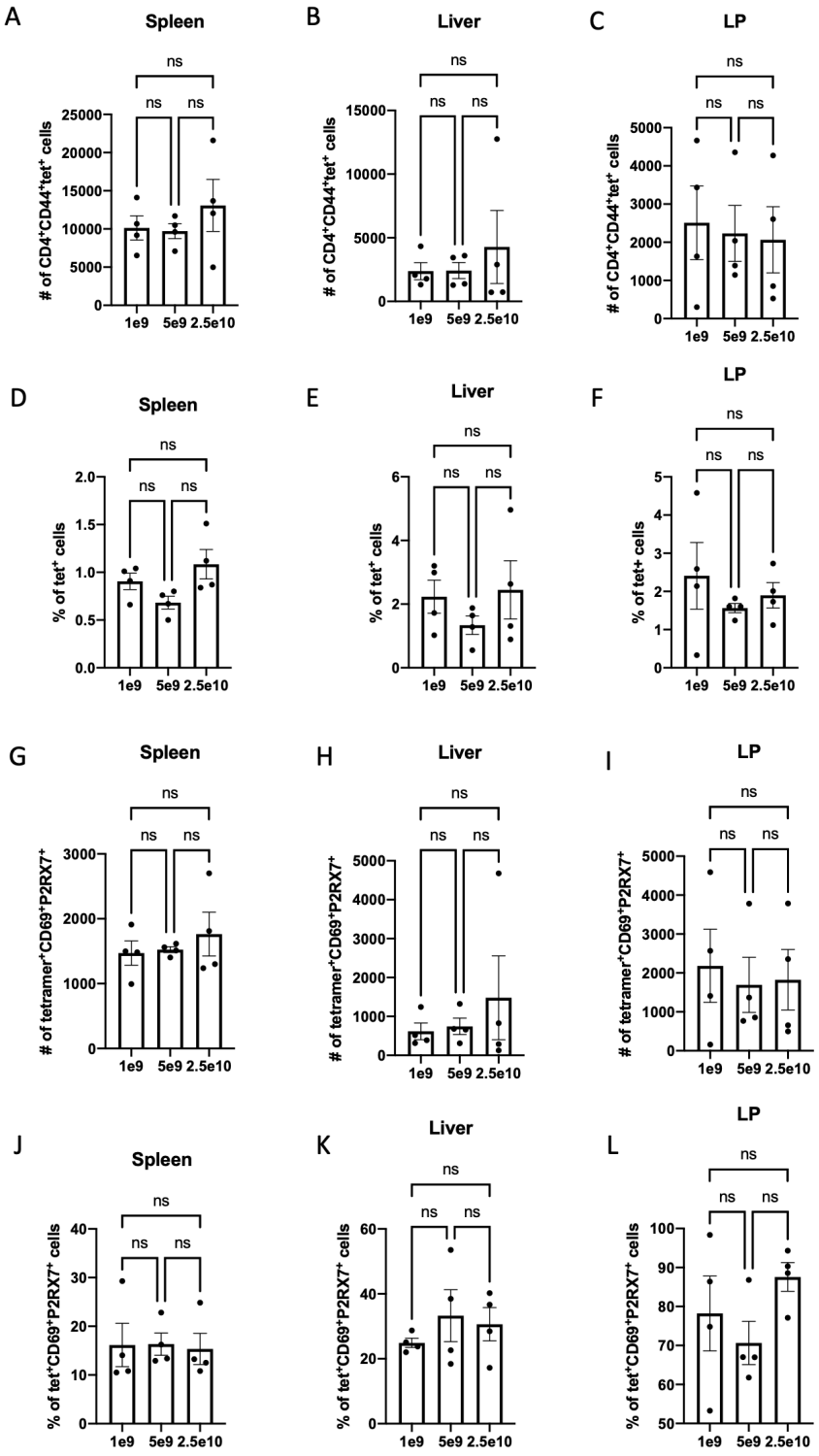


Figure 2.1: **Mucosal or systemic immunization can protect against oral or systemic infection.** C57BL/6 mice were orally (A, C-D) or intravenously (IV) (B, E-F) immunized with a live vaccine strain (LVS) of *Salmonella*. All groups were challenged with virulent *Salmonella* SL1344 orally and intravenously at 45 days post infection and protection was determined by survival (A-B) or bacterial burdens of spleen (C, E) and liver (D, F). Data are representative of two experiments with 3-4 mice in each group. Data are shown as mean ± SEM and analyzed by unpaired Student *t* test (C-F). $^{**}p \geq 0.001$, $^{***}p \geq 0.0001$.



Supplementary Figure 2.1: **Oral priming is not dose-dependent**. C57BL/6 mice were orally primed with a live vaccine strain (LVS) of *Salmonella* to compare dosages of $1x10^9$,

$5x10^9$, and $2.5x10^{10}$ CFU/mL for induction of T_{RMS} in the spleen, liver and lamina propria with n = 4 mice in each group. Data is shown as mean ± SEM.

Systemic immunization induces systemic and mucosal memory T cells

Since antibodies and CD4 T cells play distinct roles in immunity to *Salmonella* (31–33), we evaluated these responses following oral or IV immunization. Mice immunized with LVS-*Salmonella* via either route exhibited similar IgM and IgG2a responses to *Salmonella* antigens (Fig. 2.2A, B). A minimal *Salmonella*-specific IgA response was detected in serum after oral immunization, and no detectable secretory IgA was observed in fecal pellets from either group. Therefore, LVS immunization elicits comparable serum IgM and IgG responses with low IgA levels, regardless of the immunization route.

Next, we assessed the induction of *Salmonella*-specific CD4 memory T cells in systemic and mucosal tissues following IV immunization. Using a 2W1S MHC class-II tetramer, *Salmonella*-specific memory CD4 T cells were identified in mice immunized with AroA-deficient *Salmonella* expressing 2W1S (22, 25, 32). As reported previously, IV immunization generated memory CD4 T cells in the spleen, liver, and lamina propria (LP), as evidenced by an increased percentage and absolute number of tetramer$^+$CD44$^+$ CD4 T cells (Fig. 2.3A–C). In the liver and LP, most memory CD4 T cells expressed CD69 and P2RX7, markers of tissue-resident memory (T_{RM}) cells (Fig. 2.3D–F). Notably, the percentage and total number of *Salmonella*-specific T_{RMS} were significantly higher in the liver compared to the LP (Fig. 2.3D–F). Contrary to expectations, oral immunization did not significantly enhance T_{RM} formation in the LP, as the number of LP memory T cells remained low and similar to IV immunization (Fig. 2.3G, 3H). However, *Salmonella*-

specific memory T cells in the LP, regardless of the immunization route, consistently expressed CD69 and P2RX7 (Fig. 2.3I–K). These findings indicate that LVS immunization robustly induces hepatic T_{RMS} but only generates a small population of T_{RMS} in the LP, irrespective of the immunization route.

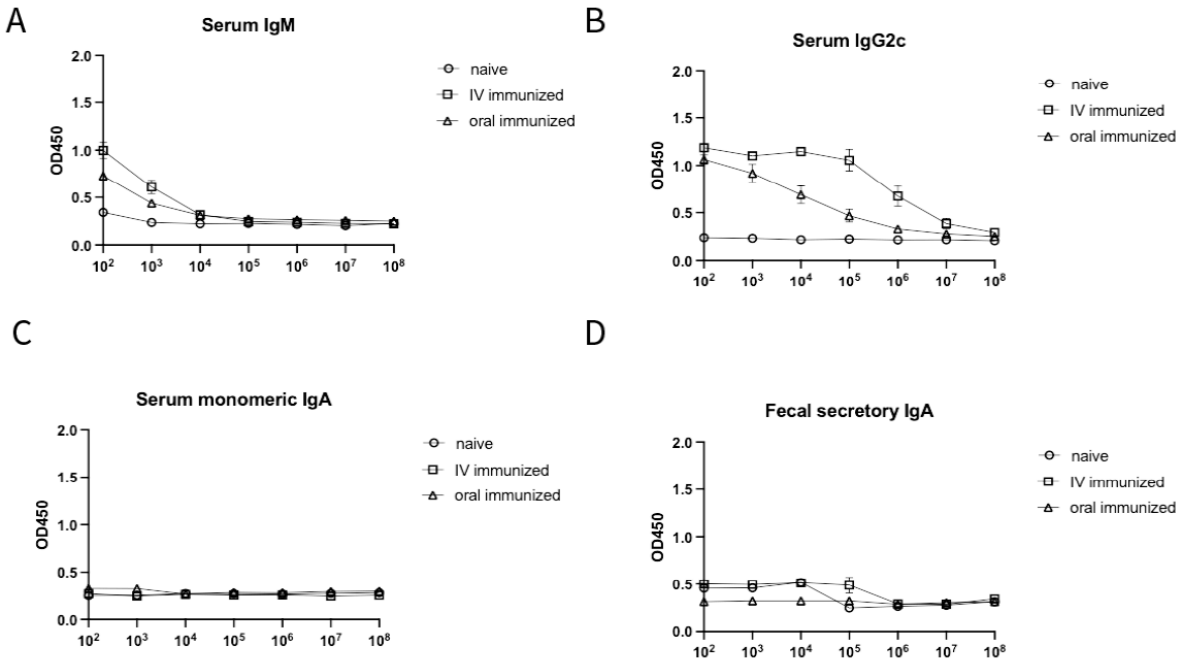


Figure 2.2: **LVS immunization elicits similar serum IgM and IgG responses, and low IgA, irrespective of route of immunization**. C57BL/6 mice were immunized orally or intravenously with *Salmonella* Typhimurium and 35 days later, serum and poop pellets were collected to determine antibody responses by ELISA. Line graphs show mean absorbance of four mice per group.

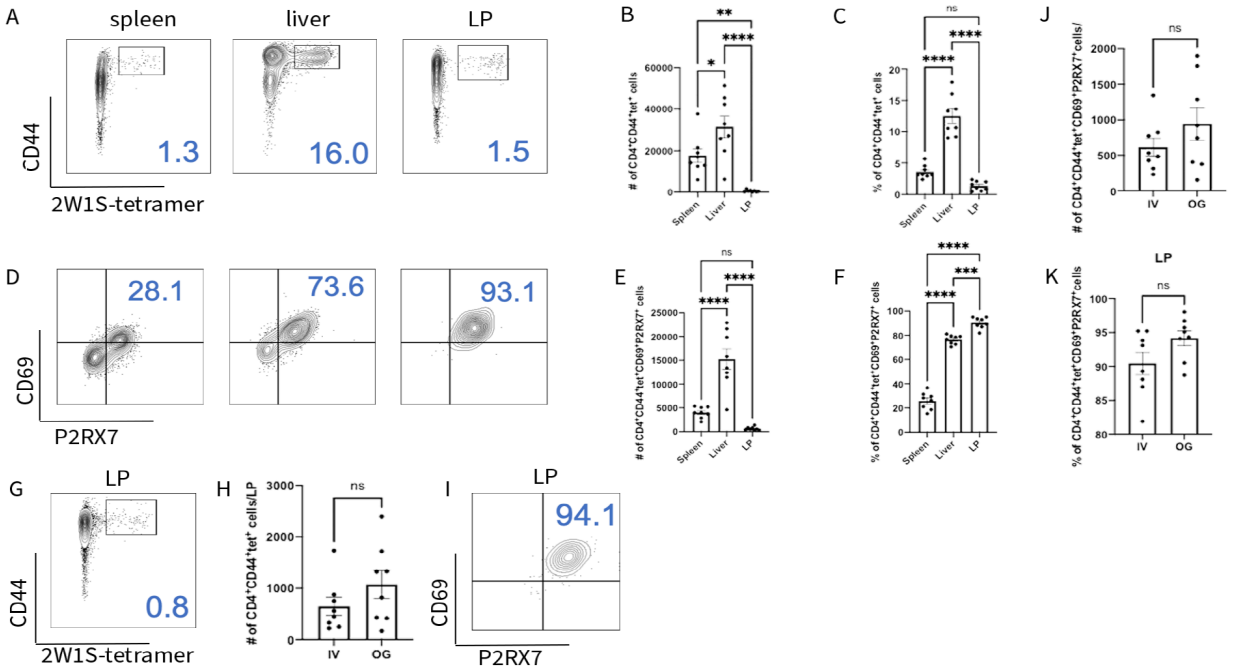


Figure 2.3: **Systemic immunization induces systemic and mucosal memory T cells.** C57BL/6 mice were immunized with *Salmonella*-2W1S-LVS intravenously (A-K) or orally (G-K) and 45 days later, spleen, liver, and lamina propria (LP) were collected and analyzed by flow cytometry. Immunization-specific CD4 T cells were identified by 2W1S-tetramer staining (A-C, G) and T_{RM} were identified as $CD69^{+}P2RX7^{+}tetramer^{+}$ CD4 T cells (D-F, I-K).

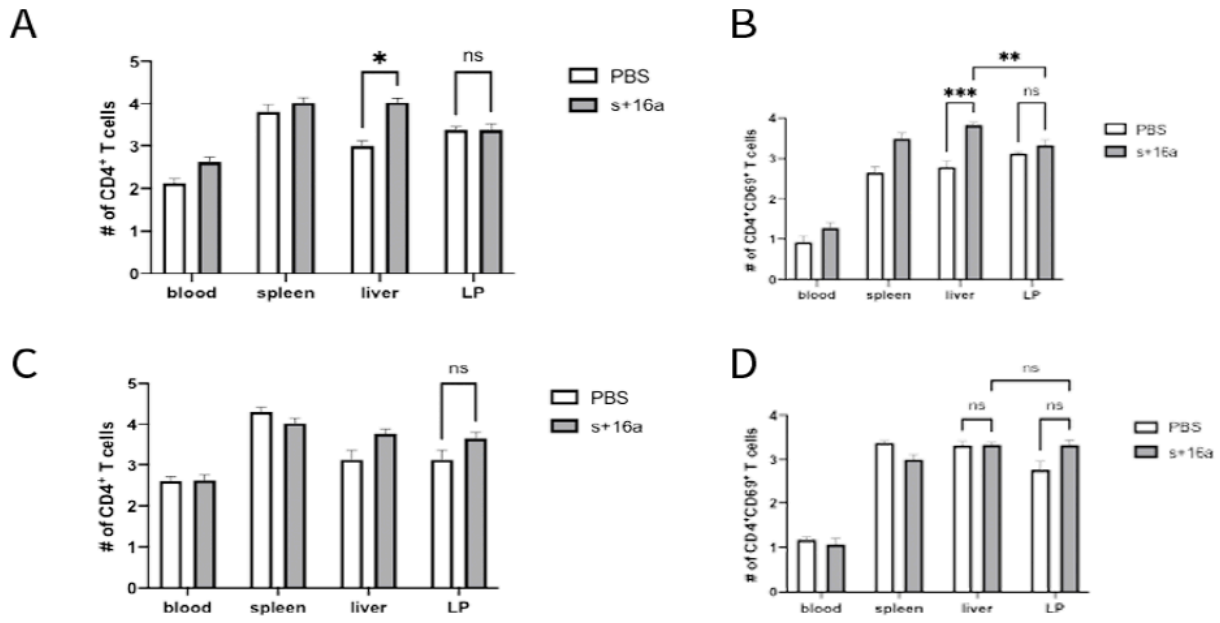
Liver T_{RM} T cells are more protective than intestinal T_{RM} *T cells*

The findings above indicate that liver T_{RMS} constitute a significant portion of the CD4 T cell memory response following LVS-*Salmonella* immunization, regardless of the immunization route. Previous studies have demonstrated that adoptive transfer of *Salmonella*-specific T_{RMS} into naïve mice confers protection against challenge infection, provided that s+16a nanobody is used to prevent T_{RM} cell death (22, 23, 26). This allowed us to directly compare the protective potential of *Salmonella*-specific T_{RMS} isolated from

the liver and the LP. In initial experiments assessing homing potential, T cells from the liver and LP of LVS-immunized mice were transferred into TCRα-deficient recipient mice, with or without s+16a to inhibit NAD-mediated cell death. Liver T_{RMS} displayed a clear preference for homing to the liver, with some also detected in the spleen and LP, especially when s+16a was used to protect them (Fig. S2.2A, B). In contrast, LP T_{RMS} were found across all tissues of the recipient mice, regardless of s+16a treatment (Fig. S2.2C, D). These results demonstrate that liver T_{RMS} exhibit a strong tendency to return to their tissue of origin, a phenomenon previously shown to depend on T-bet regulation of surface CD18 expression (26).

We next assessed the protective efficacy of liver versus LP T_{RMS}. Recipient mice were challenged either intravenously (IV) with attenuated bacteria or orally with wild-type bacteria to evaluate protection conferred by T_{RMS} from each tissue. Control mice without T cell transfer exhibited high bacterial burdens in the spleen and liver, regardless of the infection route (Fig. 2.4A–D, no transfer). In contrast, mice immunized with LVS-*Salmonella* showed a >1000-fold reduction in bacterial counts in both organs (Fig. 2.4A–D, immunized). Adoptive transfer of LP T_{RMS} reduced bacterial burdens in the liver and spleen of mice challenged IV; however, following oral challenge, this protective effect was only observed in the spleen (Fig. 2.4A–D, LP transfer). Notably, the transfer of liver T_{RMS} provided significantly stronger protection compared to LP T_{RMS}, particularly in IV-challenged mice (Fig. 2.4A–D). In contrast, for oral challenges, liver and LP T_{RMS} offered comparable levels of protection. To assess whether these differences translated into improved survival, we transferred liver or LP T_{RMS} into recipient mice and monitored survival after IV or oral challenge. Remarkably, liver T_{RM} transfer conferred protection to

over 75% of mice, irrespective of the infection route (Fig. 2.4E, F). By contrast, LP T_{RM} transfer extended survival but ultimately failed to fully protect recipient mice, regardless of whether the challenge was IV or oral (Fig. 2.4E, F). These results demonstrate that liver T_{RMS} are significantly more protective than LP T_{RMS}, regardless of whether the challenge is systemic or mucosal.



Supplementary Figure 2.2: **Liver memory cells home to the liver while LP memory cells home heterogeneously.** C57BL/6 mice were immunized with *Salmonella*-2W1S intravenously and 45 days later, received 50µg S+16a or PBS by intravenous injection 15 min before harvest of T cells isolated from the liver and LP for adoptive transfer into TCRα-deficient recipients. 24 hours later, homing of transferred lymphocytes was determined.

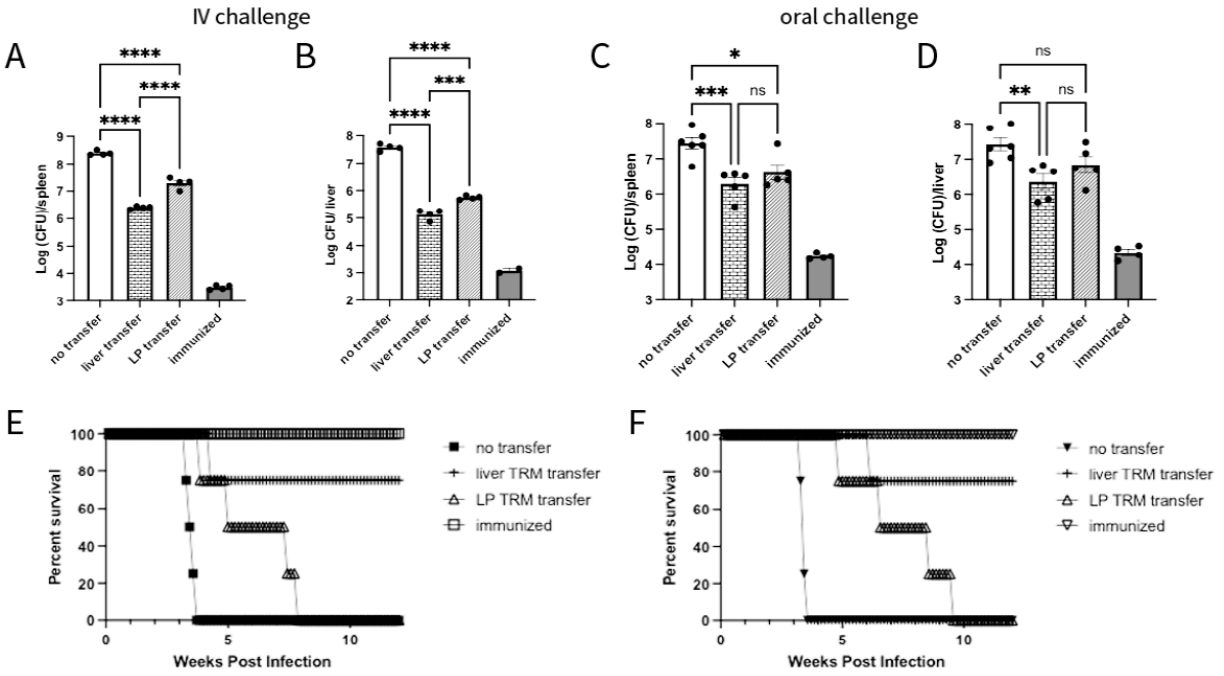


Figure 2.4: **Liver T_{RM} T cells are more protective than intestinal T_{RM} T cells.** C57BL/6 mice were immunized with *Salmonella*-2W1S intravenously and 45 days later, received 50 µg S+16a or PBS by intravenous injection 15 min before harvest of T cells isolated from the liver and LP for adoptive transfer into TCRα-deficient recipients. Recipients were challenged intravenously with attenuated *Salmonella (*A-B, E) or virulent *Salmonella* orally (C-D, F) 24h after adoptive transfer. Significance was calculated by one-way ANOVA and data are means ± SEM.

Discussion

Immunity to oral pathogens is often discussed in terms of protective host responses that develop at the intestinal mucosa (34, 35). Mouse models of *Salmonella* infection have been instrumental in elucidating the roles of M cells, Peyer's patches, IgA, and mucosal lymphocyte responses in intestinal immunity (36–39). However, while *Salmonella* enters the host via the intestinal route, the necessity of mucosal protection in

preventing infection remains unclear (29). Resolving this question is critical, as systemic Salmonellosis, caused by ingestion of contaminated food or water, claims approximately 200,000 lives annually (7, 40). Additionally, effective vaccines are lacking for most *Salmonella* infections (13). For decades, studies have shown that oral immunization with attenuated *Salmonella* provides robust protection in mice (30). This finding spurred the development of live vaccine strains (LVS) of *Salmonella*, which can be administered orally and provide moderate efficacy as vaccines (15). The assumption behind this strategy is that oral delivery is superior because it elicits sufficient mucosal immunity to protect against subsequent oral infections (14, 15). Our findings challenge this assumption. We show that systemic administration of LVS-*Salmonella* effectively protects against mucosal infection, despite failing to induce strong mucosal immune memory. This does not imply that mucosal immune responses are incapable of providing protection against oral infection; rather, it suggests that the requirement for a protective immune response within the intestine itself may be less critical than previously believed.

When analyzing the induction of mucosal and systemic immune responses following LVS immunization, we observed that T_{RMS} were generated in both the liver and lamina propria (LP), though their frequency was significantly higher in the liver. This finding aligns with a model in which most T cell clonal expansion occurs in the spleen, after which expanded memory precursors migrate to and establish residency in specific tissues. During systemic *Salmonella* infection, extensive bacterial replication occurs in the liver but not in the LP, suggesting that the higher antigen load in the liver promotes the maturation of T_{RMS} within this tissue. Additionally, the limited replication of LVS-

Salmonella in the liver likely generates the inflammatory signals necessary to support T_{RM} formation (25).

Adoptive transfer of liver T_{RMS} to immune-deficient mice conferred significant protection against infection, whereas the transfer of LP T_{RMS} did not. While this comparison is not entirely equivalent—given the differing homing properties of these populations that may affect their protective potential in a new host—both T_{RM} populations were functional in vivo, as evidenced by their ability to reduce bacterial replication when equal numbers were transferred. However, only the transfer of liver T_{RMS} improved overall survival, indicating that this population possesses superior protective capacity in vivo.

Overall, this study has important implications for the development of vaccines for systemic Salmonellosis. We find that the induction of hepatic T_{RMS} is a crucial factor in controlling systemic *Salmonella* replication, while the induction of mucosal immune responses is not essential. This conclusion makes sense, given that bacterial replication primarily occurs in the liver, not in the lamina propria. Even if mucosal immune responses are induced to control most bacteria at the site of intestinal entry, some bacteria may still reach the liver, where they can replicate and cause systemic symptoms. Vaccines targeting systemic *Salmonella* may be more effective if they prioritize parenteral administration, enhance Th1 expansion in lymphoid tissues, and promote liver memory residency. Future research will focus on the mechanism behind enhanced survival after transfer of liver T_{RMS} that suggests this population has greater protective capacity in vivo to further push along understanding and development of a parenteral *Salmonella* vaccine that stimulates robust liver T_{RM} responses.

Materials and Methods

Ethics statement

This study was conducted in full compliance with the guidelines outlined in the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. The University of California, Davis, holds accreditation from the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). All animal experiments received approval from the Institutional Animal Care and Use Committee (IACUC) at the University of California, Davis, under protocol numbers 21869 and 23467.

Mouse strains

C57BL/6 (JAX stock no. 000664), *Cd4*-cre (JAX stock no. 017336) and B6.129S2-*Tcra*^{tm1Mom}/J (JAX stock no. 002115) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) at 6-8 weeks of age and used for experiments at 7-12 weeks old and/or used for establishment of breeding colonies. All mice were genotyped before use by PCR according to protocols provided by The Jackson Laboratory and housed in specific pathogen-free conditions and cared for in accordance with the University of California, Davis Institutional Animal Care and Use Committee, and Institutes of Health guidelines.

Bacterial strains and infection

The live vaccine strain (LVS) of *Salmonella* enterica serovar Typhimurium (BMM50) was developed by the laboratory of Dr. Andreas Bäumler at University of California, Davis, through the introduction of a null mutation in the *aroA* gene of virulent *Salmonella* strain SL1344. BMM50 was subsequently modified to express a short peptide sequence (EAWGALANWAVDSA) in-frame with OmpC (BMM51), following the same method used

in our previous modification of BRD509 (22, 27). Initial experiments verified successful gene targeting, peptide expression, and the activation of 2W1S-specific CD4 T cells in vivo. Prior to use, bacteria were streaked on a MacConkey agar plate and individual colonies were used to inoculate Luria-Bertani broth (Becton Dickinson), followed by static overnight growth at 37°C and bacterial concentrations estimated using a spectrophotometer with bacteria being grown to an OD_{600} between 0.4-0.6. *Salmonella* cultures were then diluted in Phosphate Buffered Saline (PBS) to concentrations of 5×10^5 CFU/0.2mL (BMM50), 2.5×10^6 CFU/0.2mL (BMM51), or 1×10^3 CFU/0.2mL (SL1344), with 0.2mL injected via the lateral tail vein for systemic route of infection. For oral infection, an appropriate volume of overnight *Salmonella* culture was centrifuged for 30 minutes at 6000-8000 rcf at 4°C. Supernatant was discarded and bacterial pellets were resuspended in a total of 50mL of water and diluted to appropriate bacterial numbers (1×10^9 CFU) to be used to refill individual water bottles. For oral infection by gavage needle, mice were pretreated with 5% NaHCO3 solution intragastrically (ig) before being infected with *Salmonella* (5×10^9 CFU/mL) diluted in PBS. Actual concentration of bacterial dose administered was confirmed by serial dilutions and plating onto MacConkey agar plates in all experiments.

Enumeration of bacteria burden

To assess bacterial colonization in vivo, mice were euthanized via cervical dislocation and spleen, liver, and lamina propria from uninfected and infected mice were collected in 1× PBS on ice, homogenized and brought to final volumes of 2, 5 and 2mL, respectively. Samples were thoroughly mixed and serial dilutions of the homogenates were plated onto

MacConkey agar plates. Following overnight incubation at 37°C, colonies from serial dilutions were quantified and back-calculated to identify total cfu present in each organ.

Lymphocyte isolation

Spleens were collected and placed in 2% fetal bovine serum (FBS) in 1× PBS. Single-cell suspensions were prepared by pressing the spleens through a 70-μm mesh cell strainer filter unit (catalog no. 08-771-2, Fisher). The suspensions were centrifuged at 2000rpm for 5 minutes at 4°C, and the supernatant was discarded. To lyse red blood cells, the pellet was resuspended in 1mL of ammonium-chloride-potassium (ACK) lysis buffer (catalog no. A1049201, Thermo Fisher) and incubated at room temperature for 3-5 minutes. Lysis was stopped by adding 9mL of 2% FBS in 1× PBS. The suspensions were centrifuged and decanted as previously described. The pellets were then resuspended in 2% FBS in 1× PBS, and cells were quantified (Nexcelom Cytometer Auto T4 Bright Field Counter or via a hemocytometer).

Livers were collected and placed in 2% FBS in 1× PBS and single-cell suspensions were prepared as with spleens. Cellular suspensions were spun at 2000rpm for 15 minutes at room temperature with no brake. Supernatant was discarded and cells were resuspended in 15mL of 35% isotonic Percoll solution (P1644, Sigma-Aldrich). Cellular suspensions were spun at 2000rpm for 20 minutes at room temperature with no brake. Hepatocytes were gently aspirated and cellular pellets were washed in 40mL 2% FBS in 1× PBS. Red blood cells were lysed, and cells were resuspended in 2% FBS in 1× PBS and quantified. Lamina propria were collected and placed in CMF media (1× PBS, 1M HEPES, FBS, 7.5% sodium bicarbonate). Intestinal tissue is kept moist with CMF media while displacing

feces and fat tissue. Intestinal tissue is cut longitudinally and into small pieces and incubated in 10mL of DTE solution (CMF media with FBS and 100mM DTE) for 30 minutes at 37°C with stirring (550-750rpm). Tissue and DTE were transferred to a 50mL conical tube and vortexed at max speed for 1 minute then spun at 2000rpm for 3-5 minutes at room temperature. Supernatant was aspirated and tissue was washed with DTE solution. Tissue pieces were resuspended in 10mL of EDTA solution (0.5M EDTA, 1× PBS, FBS, 1M HEPES) and incubated for 30 minutes at 37°C with stirring (550-750 rpm). Pieces are then transferred to a new 50mL conical tube, spun, and washed with RPMI media (catalog no. 11875093, Thermo Fisher). Tissue pieces were then resuspended in 10mL collagenase solution (collagenase IV in 5% RPMI medium, catalog no. C818W76, Thomas Scientific) and incubated for 1hr at 37°C with stirring (650rpm) to begin tissue disaggregation. Single-cell suspensions were made by pressing tissue through a 70-μm mesh cell strainer filter unit. Cells were spun as previously described and washed in 40mL 2% FBS in 1× PBS. Pellets were suspended in 5mL of 44% Percoll solution (catalog no. 17–0891-02, GE Healthcare) and overlaid onto 3mL of 67% Percoll solution for a Percoll gradient. Cells were spun at 2000rpm for 20 min at room temperature with minimal acceleration and no break. Lymphocytes from the interface layer were removed from Percoll and washed in 10mL 2% FBS in 1× PBS. Cells were resuspended in 2% FBS in 1× PBS and then were quantified.

Adoptive transfer of lymphocytes

Liver and lamina propria of LVS-immunized mice were collected and single-cell suspensions were made as described above. Liver and lamina propria lymphocytes were

counted and equalized, then resuspended in 200μL/mouse to be transferred intravenously into a TCRα$^{-/-}$ recipient.

Antibody staining and flow cytometry

Single-cell suspensions were made as described above and 1 × 10^6 cells were incubated with Fc block (culture supernatant from 24G2 hybridoma with 2% mouse serum and 2% rat serum). Cells were incubated with Zombie Yellow (BioLegend) viability dye for 15 minutes. Cells were washed with 2% FBS in 1× PBS at 2000rpm for 5 minutes at 4°C and incubated with PE-conjugated 2W1S:I-A^b tetramer (in-house production and NIH Facility) in the presence of Fc block for an hour at room temperature in dark. Cells were washed again and stained with the following antibodies: B220, F4/80, CD11b, CD11c, NK1.1, CD4, CD44, P2RX7, CD69, CD62L, CD11a, CXCR3 (Thermo Fisher, BD Biosciences, BioLegend). Cells were washed and spun as previously described. To access cytokine production and transcription factors, cells were then permeabilized, fixed and stained in intracellular cytokine and transcription factor staining overnight using Foxp3 intra-nuclear staining kit (eBioscience). Stained cells were analyzed by flow cytometry using Becton Dickinson (BD) LSR Fortessa or BD FACSymphony with appropriate compensation and isotype controls. Flow cytometry data were analyzed with FlowJo software (TreeStar, San Carlos, CA).

Nanobody-mediated inhibition of ARTC2

For flow cytometry analysis of hepatic T cell expression of P2RX7 and transfer experiments, mice received 50μg of s+16a nanobody (catalog no. 149802, BioLegend)

diluted in 200µL of PBS, administered 15-30 minutes prior to organ collection (28).

Mouse serum and fecal samples for antibody ELISAs

Groups of C57BL/6 mice were immunized intravenously with 5×10^5 CFU/0.2 mL (BMM50) or orally with 1×10^9 CFU/water bottle. Blood and fecal pellets were collected d later. *Salmonella*-specific IgM, IgG2c and IgA responses were measured using antibody ELISA. In brief, 96-well microtiter plates were coated with heat-killed *Salmonella* and blocked with 10% FBS/PBS. Serum and fecal samples were added in serial dilutions, followed by incubation with biotin-conjugated anti-mouse IgM, IgG2c or IgA (BD Biosciences and eBioscience) and HRP-conjugated streptavidin (catalog no. N100, Thermo Fisher). The plates were developed using an HRP substrate (O-phenylenediamine dihydrochloride, Sigma-Aldrich) and analyzed with an ELISA plate reader (SpectraMax, Molecular Devices).

Statistics

Statistics were performed using GraphPad Prism version 8 (GraphPad Software, LLC). Either Mann-Whitney U test, one-way or two-way ANOVA were used. *p<0.05,**p<0.01,***p<0.01,and ****p<0.0001. Graphical figures were made using BioRender (https://biorender.com/).

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Chapter 3

Increased protective capacity of transferred T_{RMS} require IL-18R

Adapted from submission of paper:

Parenteral vaccination induces liver Tissue Resident Memory CD4 T cells and protects against mucosal *Salmonella* infection

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Introduction

The formation of immunological memory depends on the persistence of expanded memory lymphocytes following bacterial clearance (1). Antigen-primed naïve CD4 T cells differentiate into either effector or memory cells, which are distinguished by their ability to mount a rapid response upon re-exposure to the same antigen (2). After infection resolution, about 90% of effector T cells undergo apoptosis, leading to the formation of memory T cells that can self-renew and persist even without continuous peptide/MHC class-II ligand stimulation (3). These memory T cells provide a prompt effector response to previously encountered pathogens or to vaccination. As previously mentioned, CD4 Th1 cells are essential for *Salmonella* clearance during primary infection and are also crucial for protection against subsequent infections (4). Studies using parabiotic surgery and adoptive transfer to investigate T cell-mediated immunity to *Salmonella* demonstrated that both circulating (T_{EM}/ T_{CM}) and resident (T_{RM}) CD4 T cells are essential for achieving full protective immunity (5, 6).

During *Salmonella* infection, expanded *Salmonella*-specific CD4 Th1 cells release IFN-γ when they encounter infected macrophages in tissues (7). While Th1 cell activation typically occurs through TCR ligation, this effector response can still take place even without direct TCR stimulation (8). A similar process is observed during virus-specific CD8 T cell activation, where local inflammatory cytokines like IL-12 and IL-18 play a key role, although many other cytokines are involved (9, 10). In bacterial infections, inflammatory cytokines such as IL-1β and IL-18 are often produced in infected tissues due to TLR and NLR recognition of pathogen-associated molecular patterns (PAMPs) (11). This

inflammatory environment enables effector CD4 Th1 cells to detect *Salmonella*-infected tissues and produce IFN-γ in a noncognate manner (8). This innate ability of adaptive Th1 cells is critical, as *Salmonella* can effectively downregulate MHC class-II expression on macrophage surfaces (12), limiting the possibility for cognate interaction with peptide/MHC complexes. CD4 T cells can bypass this bacterial evasion strategy by responding to local inflammatory mediators like IL-18 and TL1a (8, 13). Mice with CD4 T cells that cannot respond to IL-18 or TL1a show increased susceptibility to *Salmonella*, highlighting the importance of this activation mechanism during active infection (8, 13).

We previously found that liver T_{RMS} had greater protective capacity than LP T_{RMS} and this could potentially be due to liver T_{RMS} being able to utilize IL-18 for a noncognate stimulation as we investigate this possibility.

Results

Liver T_{RM} *T cells display robust non-cognate responses*

Salmonella-specific CD4 T cells protect the host by secreting IFN-γ to activate infected macrophages (15, 16). The production of IFN-γ by Th1 cells can be further enhanced by local non-cognate (non-TCR) signals, particularly through IL-18R signaling (17, 13). We observed that IL-18R expression was higher on liver T_{RMS} compared to those in the lamina propria (LP) (Fig. 3.1A), suggesting that this cytokine receptor may enhance the protective capacity of these CD4 memory cells. To explore this, we generated a new IL-18R-floxed mouse model and crossed it with CD4-Cre mice, resulting in mice lacking IL-18R expression on T cells (Fig. 3.1B). As anticipated, CD4 T cells from CD4/IL-18R-deficient mice produced less IFN-γ upon non-cognate activation in vivo (Fig. 3.1C). These mice also had higher bacterial loads in the spleen and liver after primary *Salmonella* infection (Fig. 3.1D and 1E). Despite this deficiency in non-cognate response, CD4/IL-18R-deficient mice were eventually able to resolve the primary infection and developed similar numbers and percentages of tetramer^{+}CD44^{+} memory CD4 T cells compared to CD4Cre-negative controls (Fig. S3.1A-C). Additionally, the formation of liver T_{RMS} in these mice was comparable to controls (Fig. S3.1D-F), indicating that IL-18R is not required for the development of hepatic CD4 T_{RMS}. This allowed us to further investigate whether IL-18R is necessary for the protective function of liver or LP T_{RMS}.

Consistent with our previous findings, the adoptive transfer of Cre-/IL-18$^{fl/fl}$ liver T_{RMS} provided protection to over 80% of recipient mice from *Salmonella* infection (Fig. 3.2A). However, the transfer of liver T_{RMS} lacking IL-18R expression resulted in lower

protection against infection (Fig. 3.2A). This indicates that the ability to respond to non-cognate stimuli is crucial for liver T_{RMS} to offer full protection against *Salmonella*, in line with our data from primary infection (Fig. 3.1). As observed in the transfer experiments, the transfer of Cre-/IL-$18^{fl/fl}$ LP T_{RMS} conferred somewhat less protection than liver T_{RMS} (Fig. 3.2B). Specifically, LP T_{RMS} were only able to fully protect around 60% of recipients. Notably, this reduced protective capacity was further diminished in the absence of IL-18R (Fig. 3.2B), highlighting that LP T_{RMS} also rely on IL-18R for their modest protective function. Therefore, a key aspect of the protective ability of both liver and LP T_{RMS} is their capacity to enhance IFN-γ production through the detection of local inflammatory signals.

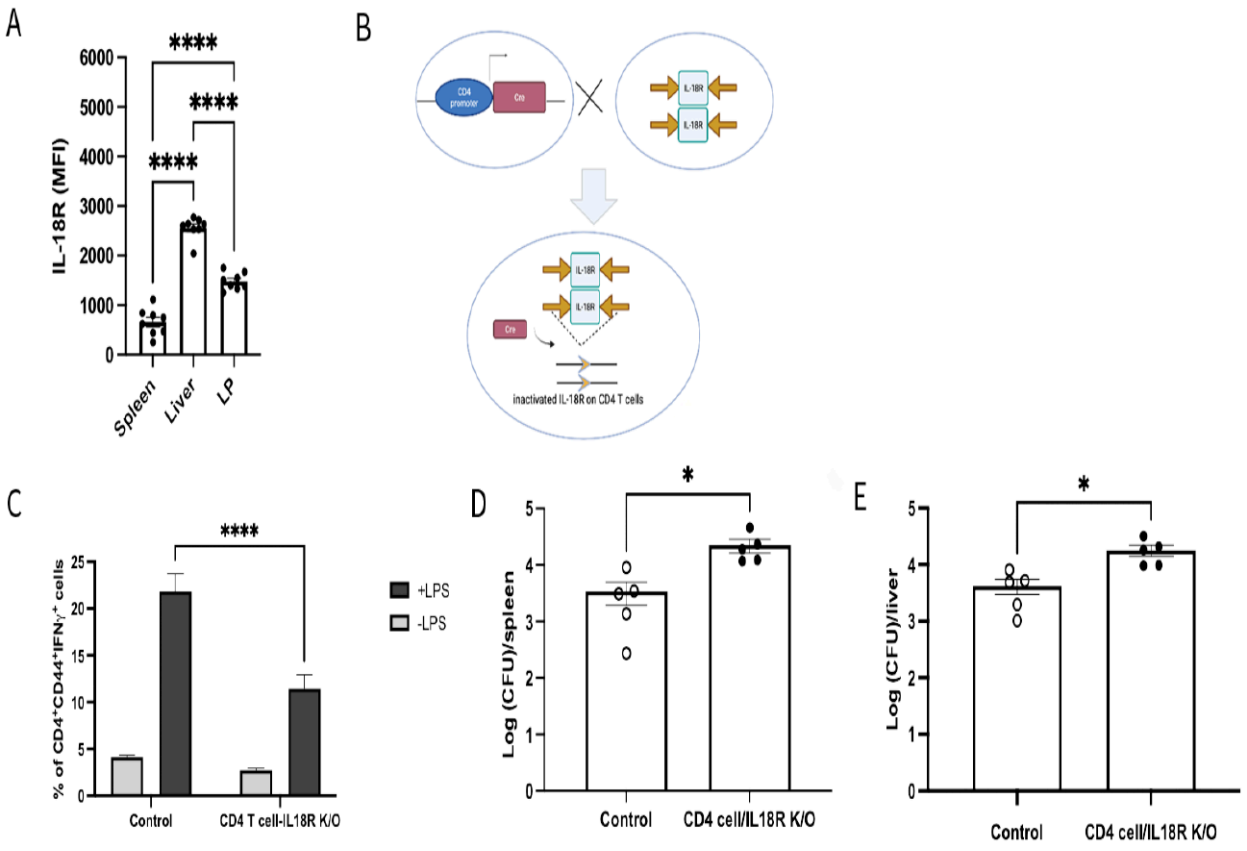
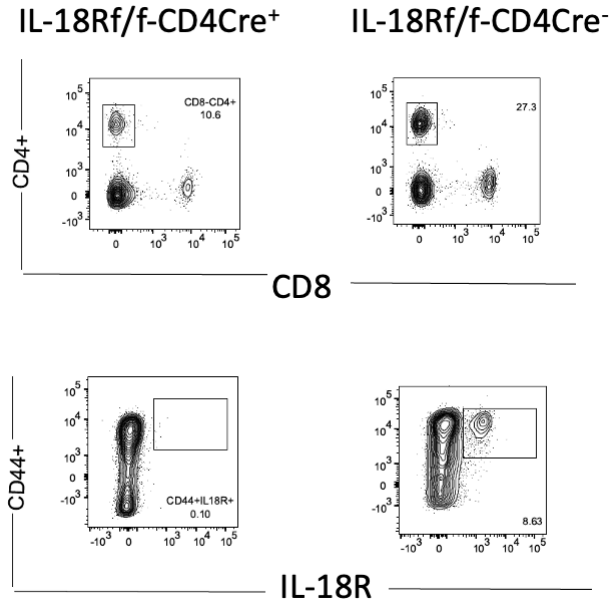
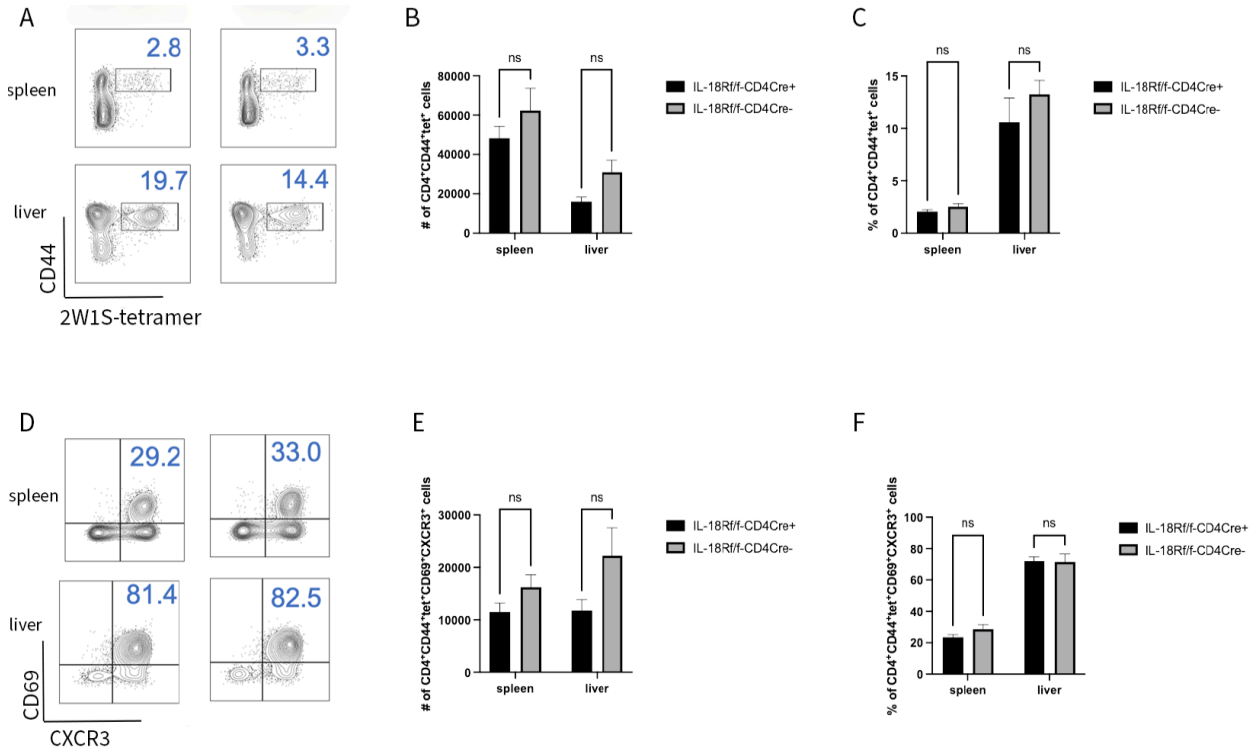


Figure 3.1: **Liver** T_{RM} **T cells display robust non-cognate responses.** C57BL/6 mice were immunized with *Salmonella*-2W1S-LVS intravenously and 45 d later, spleen, liver, and LP were collected and analyzed by flow cytometry to examine IL-18R surface marker expression (A). Schematic of an IL-18R-floxed mouse line being generated and then crossed to CD4-Cre, generating mice lacking IL-18R expression on T cells (B). CD4/IL-18R-deficient mice and littermate control were infected with LVS-*Salmonella* (C-E). Two weeks later, infected mice were injected IV with 10μg LPS and spleens were collected 4 hours later to examine IFN-γ production by Th1 cells by flow cytometry (5C). Bacterial loads in the spleen and liver of individual mice were determined at day 35 post-infection (D-E). (A) n = 4 for all experiments. Data are representative of two experiments. Significance was calculated by one-way ANOVA and data are means ± SEM. ****$p \geq$

0.0001. (C) n = 6. Significance was calculated by two-way ANOVA and data are means ± SEM. (D-E) n = 5. Data are shown as mean ± SEM and analyzed by unpaired Student *t* test, $*p \geq 0.01$.



Supplementary Figure 3.1: **Validation of IL-18Rf/f-CD4-Cre line**. CD4/IL-18R-deficient mice and littermate control has its spleens harvested and analyzed by flow cytometry to examine IL-18R expression.



Supplementary Figure 3.2: **IL-18R expression is not required for T_{RM} development.** CD4/IL-18R-deficient mice and littermate control were infected with LVS-*Salmonella* and 45 d later, spleen and liver were harvested and analyzed by flow cytometry to examine memory cell formation.

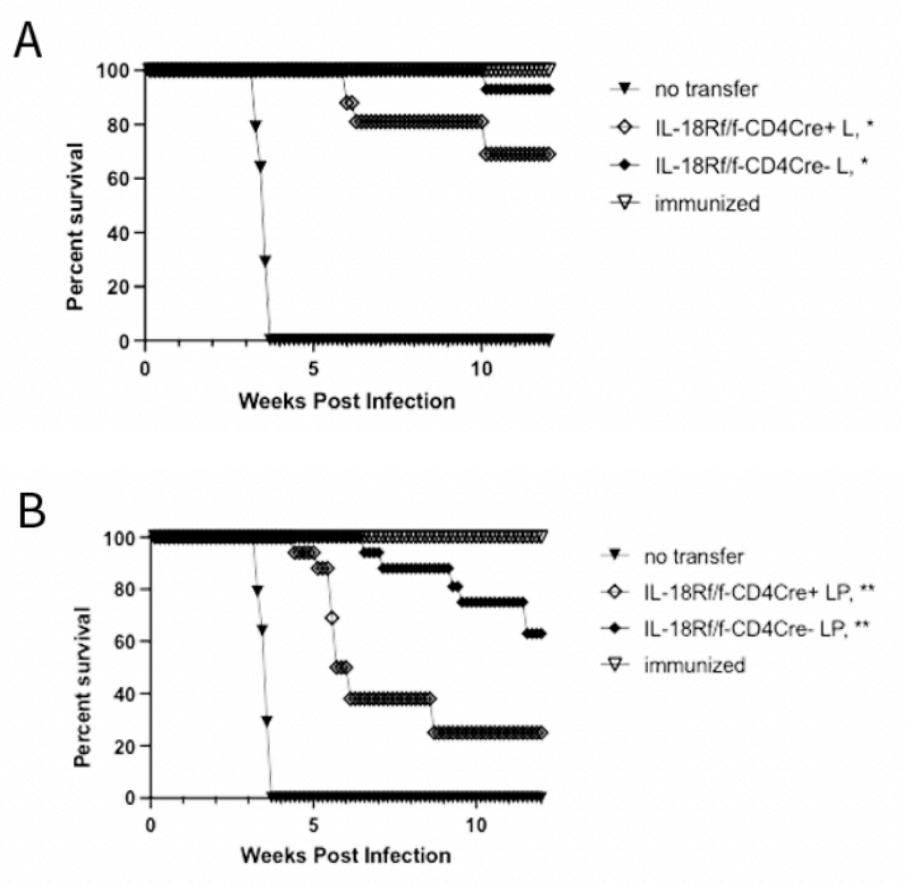


Figure 3.2: **Expression of IL-18R contributes to protection mediated by intestinal memory CD4 T cells.** CD4/IL-18R-deficient mice and littermate control were immunized with LVS-*Salmonella* and 45 d later, T cells isolated from the liver (A) and LP (B) were adoptively transferred into TCRα-deficient recipients. Recipients were challenged intravenously with attenuated *Salmonella* after adoptive transfer to determine protective capability.

Discussion

Previously, we found that adoptive transfer of liver T_{RMS} into immune-deficient mice provided significant protection against infection, whereas the transfer of LP T_{RMS} did not.

An interesting aspect of this protective effect is the necessity of IL-18R expression. Although several studies have highlighted the importance of IL-18 and non-cognate activation of CD4 T cells in immunity to *Salmonella* (17, 13, 18, 19), to our knowledge, this is the first time IL-18R has been shown to be essential for the full protective function of memory CD4 T cells. The loss of IL-18R expression reduced the protective capacity of both liver and LP T_{RMS}, underscoring the significance of this receptor in bacterial-specific memory responses. Non-cognate stimulation of CD8 T cells has been described in viral infections (10), where local inflammation amplifies effector T cell responses. While the exact reason why non-cognate activation is critical for *Salmonella* clearance remains unclear, it is possible that the inhibitory effects of *Salmonella* on MHC class-II expression allow evasion of Th1-mediated killing (20, 21). However, studies on other intramacrophage bacteria have reaffirmed the essential role of cognate T cell receptor activation (22). Future studies using bacterial mutants unable to inhibit MHC class-II expression on macrophages will likely be necessary to resolve this question.

Materials and Methods

Ethics statement

This study was conducted in full compliance with the guidelines outlined in the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. The University of California, Davis, holds accreditation from the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). All animal experiments received approval from the Institutional Animal Care and Use Committee (IACUC) at the University of California, Davis, under protocol numbers 21869 and 23467.

Mouse strains

C57BL/6 (JAX stock no. 000664), *Cd4*-cre (JAX stock no. 017336) and B6.129S2-*Tcra*^{tm1Mom}/J (JAX stock no. 002115) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) at 6-8 weeks of age and used for experiments at 7-12 weeks old and/or used for establishment of breeding colonies. T cell-specific IL-18R-deficient mice were generated for this study by intercrossing *Cd4*-cre mice and IL-18R$^{fl/fl}$ mice by the Mouse Biology Program at the University of California, Davis. All mice were genotyped before use by PCR according to protocols provided by The Jackson Laboratory and housed in specific pathogen-free conditions and cared for in accordance with the University of California, Davis Institutional Animal Care and Use Committee, and Institutes of Health guidelines.

Bacterial strains and infection

The live vaccine strain (LVS) of *Salmonella* enterica serovar Typhimurium (BMM50) was developed by the laboratory of Dr. Andreas Bäumler at University of California, Davis,

through the introduction of a null mutation in the *aroA* gene of virulent *Salmonella* strain SL1344. BMM50 was subsequently modified to express a short peptide sequence (EAWGALANWAVDSA) in-frame with OmpC (BMM51), following the same method used in our previous modification of BRD509 (6, 14). Initial experiments verified successful gene targeting, peptide expression, and the activation of 2W1S-specific CD4 T cells in vivo. Prior to use, bacteria were streaked on a MacConkey agar plate and individual colonies were used to inoculate Luria-Bertani broth (Becton Dickinson), followed by static overnight growth at 37°C and bacterial concentrations estimated using a spectrophotometer with bacteria being grown to an OD_{600} between 0.4-0.6. *Salmonella* cultures were then diluted in Phosphate Buffered Saline (PBS) to concentrations of 5×10^5 CFU/0.2mL (BMM50), 2.5×10^6 CFU/0.2mL (BMM51), or 1×10^3 CFU/0.2mL (SL1344), with 0.2mL injected via the lateral tail vein for systemic route of infection. For oral infection, an appropriate volume of overnight *Salmonella* culture was centrifuged for 30 minutes at 6000-8000 rcf at 4°C. Supernatant was discarded and bacterial pellets were resuspended in a total of 50mL of water and diluted to appropriate bacterial numbers (1×10^9 CFU) to be used to refill individual water bottles. For oral infection by gavage needle, mice were pretreated with 5% NaHCO3 solution intragastrically (ig) before being infected with *Salmonella* (5×10^9 CFU/mL) diluted in PBS. Actual concentration of bacterial dose administered was confirmed by serial dilutions and plating onto MacConkey agar plates in all experiments.

Enumeration of bacteria burden

To assess bacterial colonization in vivo, mice were euthanized via cervical dislocation and spleen, liver, and lamina propria from uninfected and infected mice were collected in 1× PBS on ice, homogenized and brought to final volumes of 2, 5 and 2mL, respectively. Samples were thoroughly mixed and serial dilutions of the homogenates were plated onto MacConkey agar plates. Following overnight incubation at 37°C, colonies from serial dilutions were quantified and back-calculated to identify total cfu present in each organ.

Lymphocyte isolation

Spleens were collected and placed in 2% fetal bovine serum (FBS) in 1× PBS. Single-cell suspensions were prepared by pressing the spleens through a 70-μm mesh cell strainer filter unit (catalog no. 08-771-2, Fisher). The suspensions were centrifuged at 2000rpm for 5 minutes at 4°C, and the supernatant was discarded. To lyse red blood cells, the pellet was resuspended in 1mL of ammonium-chloride-potassium (ACK) lysis buffer (catalog no. A1049201, Thermo Fisher) and incubated at room temperature for 3-5 minutes. Lysis was stopped by adding 9mL of 2% FBS in 1× PBS. The suspensions were centrifuged and decanted as previously described. The pellets were then resuspended in 2% FBS in 1× PBS, and cells were quantified (Nexcelom Cytometer Auto T4 Bright Field Counter or via a hemocytometer).

Livers were collected and placed in 2% FBS in 1× PBS and single-cell suspensions were prepared as with spleens. Cellular suspensions were spun at 2000rpm for 15 minutes at room temperature with no brake. Supernatant was discarded and cells were resuspended in 15mL of 35% isotonic Percoll solution (P1644, Sigma-Aldrich). Cellular suspensions

were spun at 2000rpm for 20 minutes at room temperature with no brake. Hepatocytes were gently aspirated and cellular pellets were washed in 40mL 2% FBS in 1× PBS. Red blood cells were lysed, and cells were resuspended in 2% FBS in 1× PBS and quantified.

Lamina propria were collected and placed in CMF media (1× PBS, 1M HEPES, FBS, 7.5% sodium bicarbonate). Intestinal tissue is kept moist with CMF media while displacing feces and fat tissue. Intestinal tissue is cut longitudinally and into small pieces and incubated in 10mL of DTE solution (CMF media with FBS and 100mM DTE) for 30 minutes at 37°C with stirring (550-750rpm). Tissue and DTE were transferred to a 50mL conical tube and vortexed at max speed for 1 minute then spun at 2000rpm for 3-5 minutes at room temperature. Supernatant was aspirated and tissue was washed with DTE solution. Tissue pieces were resuspended in 10mL of EDTA solution (0.5M EDTA, 1× PBS, FBS, 1M HEPES) and incubated for 30 minutes at 37°C with stirring (550-750 rpm). Pieces are then transferred to a new 50mL conical tube, spun, and washed with RPMI media (catalog no. 11875093, Thermo Fisher). Tissue pieces were then resuspended in 10mL collagenase solution (collagenase IV in 5% RPMI medium, catalog no. C818W76, Thomas Scientific) and incubated for 1hr at 37°C with stirring (650rpm) to begin tissue disaggregation. Single-cell suspensions were made by pressing tissue through a 70-μm mesh cell strainer filter unit. Cells were spun as previously described and washed in 40mL 2% FBS in 1× PBS. Pellets were suspended in 5mL of 44% Percoll solution (catalog no. 17–0891-02, GE Healthcare) and overlaid onto 3mL of 67% Percoll solution for a Percoll gradient. Cells were spun at 2000rpm for 20 min at room temperature with minimal acceleration and no break. Lymphocytes from

the interface layer were removed from Percoll and washed in 10mL 2% FBS in 1× PBS. Cells were resuspended in 2% FBS in 1× PBS and then were quantified.

Adoptive transfer of lymphocytes

Liver and lamina propria of LVS-immunized mice were collected and single-cell suspensions were made as described above. Liver and lamina propria lymphocytes were counted and equalized, then resuspended in 200µL/mouse to be transferred intravenously into a TCRα$^{-/-}$ recipient.

In vivo stimulation with LPS

Ultrapure LPS from E.coli strain EH100Ra (TLRgrade, Enzo) was diluted in 1× PBS (UltraPure PBS, GIBCO) and injected intravenously at a dose of 10µg/mouse. Spleens and livers were harvested from uninfected and infected mice 4 hours after LPS administration and processed for flow cytometric analysis of cytokine production.

Antibody staining and flow cytometry

Single-cell suspensions were made as described above and 1 × 10^6 cells were incubated with Fc block (culture supernatant from 24G2 hybridoma with 2% mouse serum and 2% rat serum). Cells were incubated with Zombie Yellow (BioLegend) viability dye for 15 minutes. Cells were washed with 2% FBS in 1× PBS at 2000rpm for 5 minutes at 4°C and incubated with PE-conjugated 2W1S:I-A^b tetramer (in-house production and NIH Facility) in the presence of Fc block for an hour at room temperature in dark. Cells were washed again and stained with the following antibodies: B220, F4/80, CD11b, CD11c, NK1.1, CD4, CD44, P2RX7, CD69, CD62L, CD11a, CXCR3, IL-18 (Thermo Fisher, BD

Biosciences, BioLegend). Cells were washed and spun as previously described. To access cytokine production and transcription factors, cells were then permeabilized, fixed and stained in intracellular cytokine and transcription factor staining overnight using Foxp3 intra-nuclear staining kit (eBioscience). Stained cells were analyzed by flow cytometry using Becton Dickinson (BD) LSR Fortessa or BD FACSymphony with appropriate compensation and isotype controls. Flow cytometry data were analyzed with FlowJo software (TreeStar, San Carlos, CA).

Nanobody-mediated inhibition of ARTC2

For flow cytometry analysis of hepatic T cell expression of P2RX7 and transfer experiments, mice received 50µg of s+16a nanobody (catalog no. 149802, BioLegend) diluted in 200µL of PBS, administered 15-30 minutes prior to organ collection (28).

Statistics

Statistics were performed using GraphPad Prism version 8 (GraphPad Software, LLC). Either Mann-Whitney U test, one-way or two-way ANOVA were used. *p<0.05,**p<0.01,***p<0.01,and ****p<0.0001. Graphical figures were made using BioRender (https://biorender.com/).

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Chapter 4

Role of IL-18R during *Chlamydia* infection

Introduction

T cells have been shown to respond to non-cognate stimuli, allowing them to secrete cytokines in response to local innate stimuli without the presence of an antigen (1). Our study revealed that protective effects of both liver and LP T_{RMS} during *Salmonella* infection relied on IL-18R expression and their capacity to generate a non-cognate response in vivo. Mice with CD4 T cells unable to respond to IL-18 or TL1a exhibit increased susceptibility to *Salmonella*, highlighting the critical role of this activation mechanism during active infection (1, 2). But does this hold true for other intracellular bacteria?

Chlamydia trachomatis is a gram-negative obligate intracellular bacteria and is the leading bacterial sexually transmitted infection, with approximately 1.6 million cases reported in the United States alone in 2021 (3). This persistent bacterial infection can cause significant pathology at the musical surfaces of epithelial cells in the urogenital tract, potentially resulting in severe reproductive complications in otherwise healthy individuals (3, 4, 5). Due to the asymptomatic nature of *Chlamydia* infections, detection and treatment are often delayed, contributing to its high incidence rate. As a result, the development of an effective vaccine has become a critical public health priority (6).

Mouse models utilizing *Chlamydia muridarum* initiates an ascending reproductive tract infection of epithelial cells, causing severe pathology similar to human *Chlamydia trachomatis* infection, thus this mouse model has been used to study the basic immunity to *Chlamydia* infections (5, 7-8). Research has demonstrated that while Th1 cells are not strictly required for clearing *Chlamydia* infection, mice lacking IFN-γ or its receptor (IFN-γR) exhibit fatal systemic infection (9). Furthermore, T-bet-deficient mice treated with

depleting anti-IFN-γ antibodies also experienced bacterial dissemination and mortality (9) and IL-12p40-dependent protective CD4 module emerges to combat *Chlamydia* infection (10). These findings highlight the critical role of IFN-γ and IL-12p40-dependent mechanisms in resolving systemic infection (9-10).

In a cascade-like function, IL-12p40 activates STAT4 via induction of tyrosine phosphorylation and DNA binding, a signaling transducer protein that facilitates T cells maturation (11). Studies have shown that STAT4 enhances differentiation of $CD103^{-}$ T_{RMS} by directly regulating genes such as *Ifng, S1pr1* and *Il18r1* (12). Based on this, we conducted a preliminary pilot experiment to investigate whether IL-18R expression plays a role during *Chlamydia* infection.

Results

Expression of IL-18R is not required for clearance from the reproductive tract

CD4-Cre *IL-18R*f/f mice resolved *Chlamydia* infection with similar kinetics to Cre-negative littermate controls with TCRα mice were severely deficient in clearing bacteria (Fig. 4.1). This data demonstrates that IL-18R expression is not required to resolve primary *Chlamydia* infection.

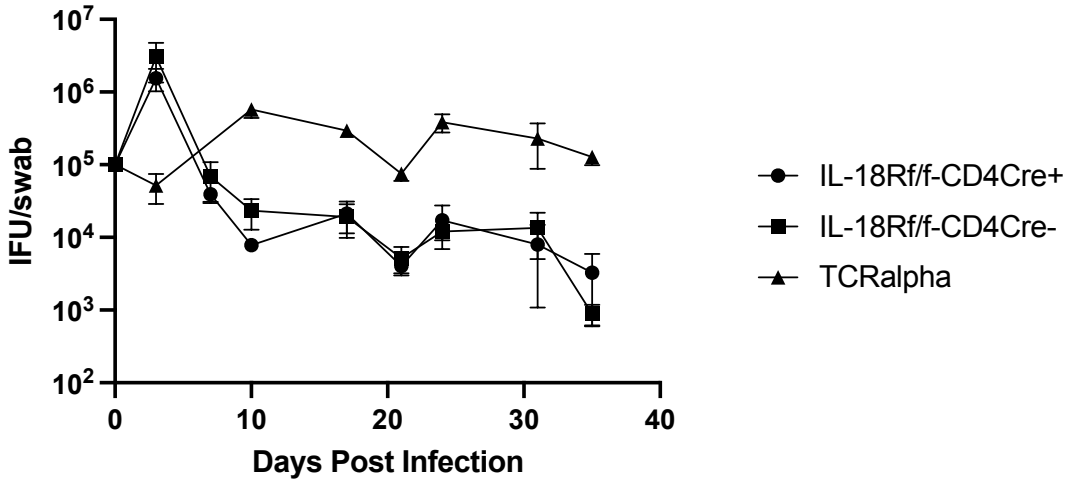

Figure 4.1. **Expression of IL-18R is not required for clearance from the reproductive tract.** Mice received 2.5mg Depo-Provera 7 days before infection with $1x10^5$ IFU *Chlamydia muridarum* intravaginally. IFU/vaginal swab over time, n = 6 for all groups and shown as mean ± SEM.

Discussion

CD4 Th1 cells utilize a highly specific T cell receptor to recognize infected cells and initiate pathogen clearance through IFN-γ secretion. Mice lacking T cell-intrinsic expression of MyD88, a key adapter molecule in non-cognate T cell stimulation, showed higher bacterial burdens when infected with *Salmonella*, *Chlamydia*, or *Brucella*. This indicates that non-cognate Th1 stimulation is a crucial component of effective bacterial clearance (2).

Our previous findings demonstrated that T_{RMS} require IL-18R for enhanced protection during *Salmonella* infection. Without IL-18R expression, IFN-γ production during primary *Salmonella* infection is significantly reduced. While *Chlamydia* relies on IFN-γ and IL-12p40-dependent mechanisms for resolving systemic infection—with IL-12p40 directly regulating IL-18R via STAT4—IL-18R expression on CD4 T cells has been shown not to be essential for *Chlamydia* clearance.

This discrepancy could be explained by the fact that *Chlamydia* is often classified as a “Th1 pathogen” due to prominent CD4 T cell IFN-γ production and the high susceptibility of IFN-γ-deficient mice (10, 14-17). However, research has shown that mice lacking T-bet can resolve female reproductive tract (FRT) infections with near-normal kinetics, suggesting that *Chlamydia muridarum* infections are not primarily controlled by classical T-bet-dependent Th1 cells in this model (9). Although IL-18R expression may still play a role, it may not be involved in the pathway mediated by classical T-bet Th1 cells for the rapid clearance of *Chlamydia* infection.

Materials and Methods

Ethics statement

This study was conducted in full compliance with the guidelines outlined in the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. The University of California, Davis, holds accreditation from the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). All animal experiments received approval from the Institutional Animal Care and Use Committee (IACUC) at the University of California, Davis, under protocol numbers 21869 and 23467.

Mice

C57BL/6 (JAX stock no. 000664), *Cd4*-cre (JAX stock no. 017336) and B6.129S2-*Tcra*^{tm1Mom}/J (JAX stock no. 002115) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) at 6-8 weeks of age and used for experiments at 7-12 weeks old and/or used for establishment of breeding colonies. T cell-specific IL-18R-deficient mice were generated for this study by intercrossing *Cd4*-cre mice and IL-18R$^{fl/fl}$ mice by the Mouse Biology Program at the University of California, Davis. All mice were genotyped before use by PCR according to protocols provided by The Jackson Laboratory and housed in specific pathogen-free conditions and cared for in accordance with the University of California, Davis Institutional Animal Care and Use Committee, and Institutes of Health guidelines.

Chlamydia infection and bacterial shedding

Chlamydia muridarum was purchased from ATCC (Manassas, VA) and propagated following established protocols (13). The estrous cycle of the mice was synchronized by administering 2.5mg of medroxyprogesterone acetate (Depo-Provera) subcutaneously one week prior to infection. For vaginal infections, $1x10^5$ IFU of *Chlamydia muridarum* in SPG buffer was pipetted into the vaginal cavity of the mice.

To evaluate in vivo bacterial shedding, vaginal swabs were collected and placed into 500 µL of SPG buffer along with two glass beads in 2 mL microcentrifuge tubes. The tubes were then shaken at 1400 rpm for 5 minutes at 4°C, after which the swab was removed. A serial dilution of the bacterial suspension was used to infect a monolayer of HeLa cells in 96-well plates. The bacteria were allowed to form inclusions overnight, then fixed, stained, and counted to determine the IFU per swab.

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Chapter 5

Conclusions

Adapted from submission of paper:

Parenteral vaccination induces liver Tissue Resident Memory CD4 T cells and protects against mucosal *Salmonella* infection

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Conclusions

Immunity to oral pathogens is frequently explored through the lens of host defenses established at the intestinal mucosa (1-2). Mouse models of *Salmonella* infection have provided valuable insights into the roles of M cells, Peyer's Patches, IgA production, and mucosal lymphocyte activity in shaping these defenses (3-6). However, despite *Salmonella* entering the host through the oral cavity and traveling to the intestinal tract, the precise role of mucosal immunity in preventing or providing protection against infections is less understood (7). Addressing this knowledge gap is crucial as systemic Salmonellosis, contracted through contaminated food or water, continues to claim approximately 200,000 lives worldwide each year (8-9). Currently, effective vaccines are unavailable for most *Salmonella* infections (10). Research has shown that oral immunization of mice with attenuated *Salmonella* provides robust protection against infection, leading to the development of live vaccine strains (LVS) which can be orally administered and show moderate effectiveness as vaccines (11). The rationale behind this approach is that oral delivery is advantageous because it can generate robust mucosal immunity, providing protection against subsequent oral infections (11-12). However, our study challenges this no assumption, as systemic administration of LVS effectively protected against mucosal infection despite failing to generate strong mucosal immune memory. These findings suggest that the protective mucosal immune response within the intestine is less clear than expected.

Following LVS immunization, we observed that tissue-resident memory cells, the first line of defense against reinfection, were generated in both the liver and lamina propria (LP). However, their frequency was significantly higher in the liver. This observation aligns

with a model in which the majority of T cell clonal expansion occurs in the spleen, followed by the migration of expanded memory precursors to specific tissue sites. Given that extensive bacterial replication occurs in the liver but not in the LP during systemic *Salmonella* infection, the antigen load in the liver likely promotes the maturation of T_{RMS} in this tissue. Additionally, the limited replication of LVS *Salmonella* in the liver likely provides the necessary inflammatory signals to support T_{RM} formation (13). The adoptive transfer of liver T_{RMS} to immune-deficient mice provided significant protection against infection, whereas the transfer of LP T_{RMS} did not. This comparison, however, is not entirely straightforward, as these two T_{RM} populations possess different homing properties that may influence their effectiveness in a new host. Nevertheless, when equal numbers of T_{RMS} were transferred, both populations reduced bacterial replication, demonstrating their functionality in vivo. Despite this, only liver T_{RM} transfer improved overall survival, highlighting the superior protective capacity of this population in vivo.

A key factor in this protection appears to be the expression of IL-18R. While previous studies have emphasized the role of IL-18 and non-cognate CD4 T cell activation in immunity against *Salmonella* (14-17), this is the first instance, to our knowledge, where IL-18R has been shown to be critical for the full protective capability of memory CD4 T cells. The absence of IL-18R expression diminished the protective potential of both liver and LP T_{RMS}, underscoring the importance of this receptor in memory responses against bacteria. Non-cognate activation of CD8 T cells has been well-documented in viral infections (18), where inflammatory signals can enhance effector T cell responses. Why non-cognate activation is crucial for *Salmonella* clearance remains unclear. One possibility is that *Salmonella*'s ability to inhibit MHC class-II expression allows it to evade

Th1-mediated killing (19-20). However, studies on other intracellular bacteria have demonstrated the essential role of cognate T cell receptor activation in bacterial clearance (21). Future research involving bacterial mutants incapable of suppressing MHC class-II expression in macrophages will be necessary to clarify this mechanism.

This prompted us to investigate the role of IL-18R expression during *Chlamydia* infection, another intracellular bacterium previously shown to exhibit increased bacterial burdens in the absence of MyD88 (15). Additionally, IL-12p40, known to be critical for bacterial clearance, plays a role in activating STAT4, which regulates IL-18R expression (22-24). Preliminary data suggests that IL-18R expression is not essential for *Chlamydia* clearance. However, this finding is nuanced by the fact that *Chlamydia* does not appear to be resolved through a classical Th1-mediated pathway.

Overall, our study provides valuable insights for the development of vaccines targeting systemic Salmonellosis. We demonstrate that the induction of hepatic T_{RMS} is essential for controlling systemic *Salmonella* replication, whereas mucosal immune responses appear to be non-essential. This finding aligns with the observation that bacterial replication occurs primarily in the liver rather than the lamina propria. Even if mucosal responses were effective in reducing bacterial load upon intestinal entry, any bacteria that manage to reach the liver could still replicate and trigger systemic disease. Therefore, vaccines designed to combat systemic *Salmonella* may benefit from a focus on parenteral administration to optimize Th1 expansion in lymphoid tissues and promote liver-resident memory T cell formation. Future research will aim to develop a parenteral *Salmonella* vaccine capable of eliciting robust hepatic T_{RM} responses.

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