HOW SALMONELLA INFECTION CAN INFORM ON MECHANISMS OF IMMUNE FUNCTION AND HOMEOSTASIS

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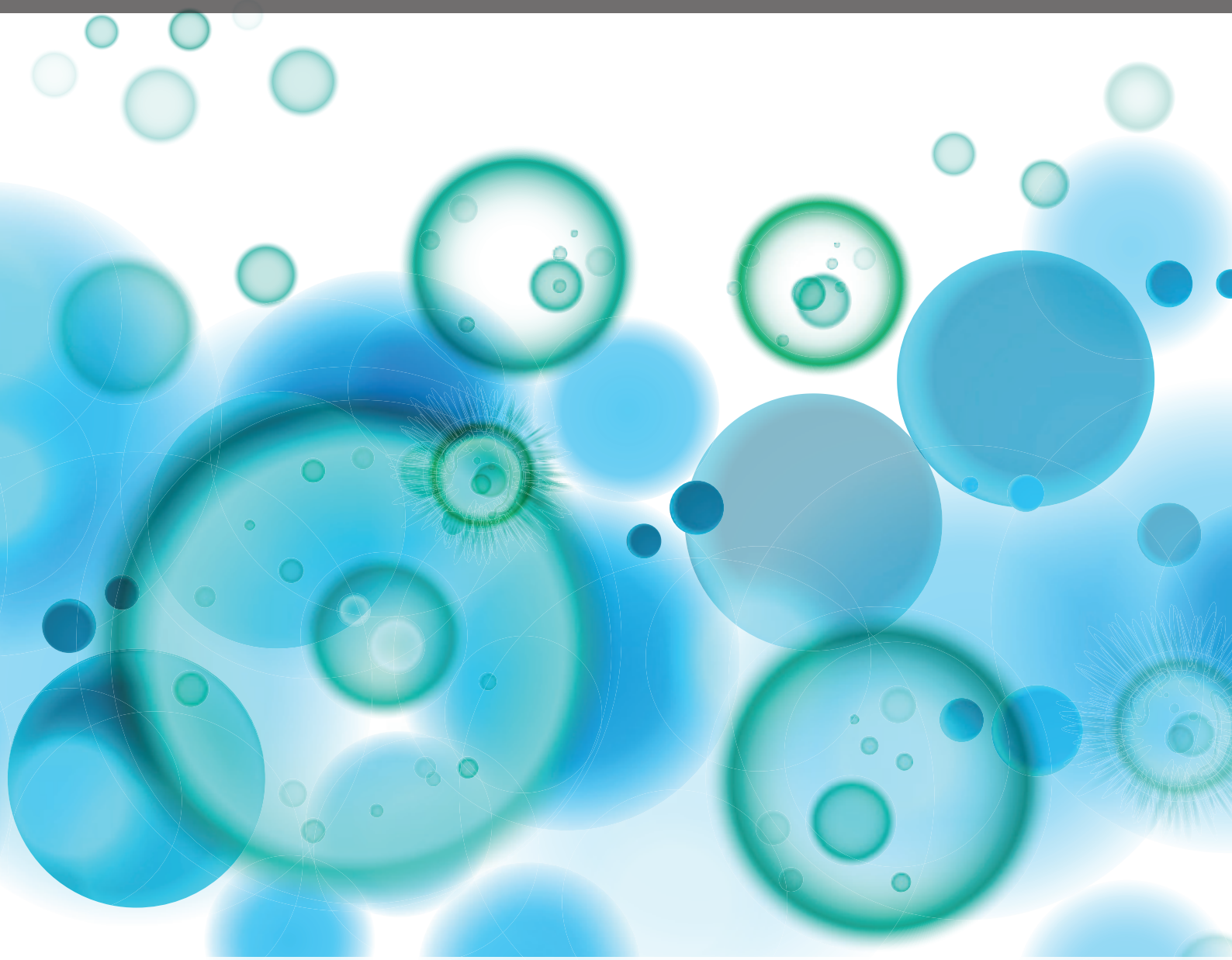

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HOW SALMONELLA INFECTION CAN INFORM ON MECHANISMS OF IMMUNE FUNCTION AND HOMEOSTASIS

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The use of model antigens such as haptens and ovalbumin has provided enormous insights into how immune responses develop, particularly to vaccine antigens. Furthermore, these studies are overwhelmingly performed in animals housed in clean facilities and are not known to have experienced overt clinical signs caused by infectious agents. Therefore, this is unlikely to reflect the impact more complex host-pathogen interactions can have on the host, nor the diversity in how immunity is regulated. Humans develop immune responses in the context of the periodic exposure to multiple pathogens and vaccines over a life-time. These are likely to have a long-lasting effect on who and what we are and how we respond to further antigen challenge. Therefore, studies on how infection influences immune homeostasis and how the development of responses to a pathogen reflects what is known on immune regulation will be informative on how we can translate findings from our standard models into treatments usable in humans.

One organism allows us to do just this. Bacteria of the genus Salmonella are devastating human pathogens. Nevertheless, many aspects of the diseases they cause can be successfully modelled in murine systems so that the infection is either resolving or non-resolving. This has the advantage of allowing the long-term impact of infection on immune function to be assessed. We propose to welcome key workers to write about their research that examine the consequence of Salmonella infection on the host and the elements of the bacterium that contribute to this.

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EDITORIAL
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Editorial: How *Salmonella* infection can inform on mechanisms of immune function and homeostasis

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Keywords: *Salmonella*, host responses, microbiota, innate immune system, adaptive immune system

Our ability to survive requires the competency to control infection. In the last 50 years, there has been an explosion in our understanding of the processes that underlie this. Central to this is our ability to restrict the infection to local sites and so prevent it from becoming systemic. Infections caused by serovars of the species *Salmonella enterica*, spread through fecal–oral transmission, exemplify this and are a major reason why this organism was chosen as the theme of this Research Topic. *Salmonella* infections, particularly typhoidal infections, have had their hand on the tiller of human history, able to steer fate in new directions as a consequence of their deadly properties. A key element of this is the ability to spread through the host and this is often associated with the capacity to cause fatal infections. The prevention of infection and the control of bacterial spread require the complex interplay between the microbiota and innate and adaptive immune mechanisms. The effects of *Salmonella* infections on the complex systems that regulate their control can leave short- and long-term footprints on the homeostatic functions of the host, for instance in the thymus and bone marrow (1, 2), broadens the significance of their study. The depth of interest in this organism is represented in this Research Topic.

The reasons behind the diverse clinical manifestations of this infection are introduced by Gal-Mor and colleagues (3), who discuss the differences between typhoidal and non-typhoidal *Salmonella* strains. This overview includes introducing antigens, including Vi capsule, which can be differentially expressed, as well as the distinct immune responses induced by different serovars. Whilst most groups focus their studies on *Salmonella* infections in mammalian hosts, it should be remembered that many serovars can colonize other organisms too, and indeed this provides a reservoir for most non-typhoidal strains. Wigley highlights the importance of *Salmonella* infection in chickens, both as a source of zoonotic infection, but also as a disease in itself and one of major economic importance (4). Furthermore, we can learn so much from this system, for instance chickens lack lymph nodes, have different MHC and TLR usage, and lack IgG subclasses, so the regulation of the immune response is likely to have multiple unique features. Although the severity of *Salmonella* infections in humans and mice is associated with its systemic spread, there is obviously a close relationship with the gut, well described as "a mucosal pathogen with a systemic agenda" (5). In immunological terms, this is a fascinating relationship to study. In many ways, a primary aim of the mucosal system is to limit inflammation to maintain barrier integrity, whereas systemic immunity often dramatically exploits inflammation to contain infection. This is neatly exploited by non-typhoidal *Salmonella* strains that are commonly associated with gastrointestinal infection and inflammation. In addition, Vi-expressing S. Typhi may also exploit lower levels of mucosal inflammation to help it spread throughout the host (6). Several works in this edition refer to relationship between the pathogen and the gut. Santos examines the three-way relationship between *Salmonella*, the microbiota, and the innate immune system, with a particular emphasis on how the microbiota can buffer against infection (7). Patel and McCormick further develop this concept to encompass details on the ability of *Salmonella* to exploit innate barriers and immune cells via type III secretion systems to establish

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and cause infections (8). This theme is explored in greater depth from a Salmonella perspective by Hurley and colleagues (9), who provide insights into the repertoire of virulence-associated genes used by *Salmonella* during infection. *Salmonella* pathogenicity islands (SPI) are important foci of genes that contribute to virulence. How expression of genes within these SPI is controlled is incompletely understood, particularly for the regulation of expression by transcriptional factors encoded outside the regions themselves. Guadarrama and colleagues discuss the potential role of the transcriptional global regulator LeuO in this process (10). More details of innate-like immune cells at the gut mucosa, and how they function, are provided by Ussher et al. (11), who describe the known and potential roles of mucosal-associated innate-like T cells in *Salmonella* and other infections. One important mechanism used to control intracellular survival, for instance through sensing for bacteria, is the ubiquitin pathway, control of which is a melee which ensues between the host and pathogen. How the host uses this pathway and how *Salmonella* impacts upon it is the subject of a review by Narayanan and Edelmann (12) and they also describe how understanding these interactions helps improve our comprehension of the ubiquitin pathway in health in addition to disease. The reasons underlying genetic susceptibility to *Salmonella* infections are multiple and complex. In a primary research paper, Khan et al. describe susceptibility loci in a non-standard mouse model derived from wild mice (13). This work characterizes the *immunity to typhimurium 3* (*ity3*) locus in greater detail to understand the nature of resistance to this infection.

When barriers and innate immunity are insufficient to control infection in mice and humans, then adaptive immunity, including T cells and antibody responses, can contribute to pathogen control to varying degrees. A number of papers in this Research Topic examine the role of adaptive immune responses in the generation of immunity to *Salmonella* and different strategies for vaccine development. O'Donnell and McSorley (14) expand our current appreciation of the role of T cells against bacteria. They do this by examining the development of classical T helper 1 responses to bacterial antigens, but also examine innate-like T cell activation and bystander T cell activation. Furthermore, they discuss the roles and importance of these cells and how infection can subvert their activities. CD4 T cells and the Th1 cells are most associated with control of *Salmonella* infections. Nevertheless, CD8 T cells and B cells are likely to be important too. The relationship between these latter two cell types is examined by Lopez-Medina and colleagues (15). They assess whether infection of B cells results in cross-talk between these cells and CD8 T cells and the potential for this to influence the generation of immunity. This has not been widely explored in the literature and may indicate a role of B cells for aiding in the dissemination of infection. A broad assessment of the immune response to typhoid in humans is provided by Sztein and colleagues (16) and covers T and B cell responses to active infection and the major antigens recognized by the host during infection. A key focus of this study and the central point of the article by Jones et al. is the reintroduction of the human challenge model for typhoid (17, 18). The ability to know the exact time an individual is infected overcomes a major complication in the study of this disease, which is identifying the stage after infection a response is being measured. This should help to identify improved ways to diagnose infection and protect against it through vaccination.

Typhoid is unusual in that there are three vaccines that provide similar protection against disease, albeit that the protection is limited and relatively short-lived, reflecting the efforts employed to limit its spread. Bumann, MacLennan, and Sztein et al. (16, 19, 20) examine the mechanisms of protection against infection through vaccination. Sztein et al. describe many studies using live-attenuated vaccines against typhoid, the lessons that have been learned and the potential for conjugate vaccines generated around the Vi antigen. Bumann focuses on identifying the properties of antigens and how to identify those with the potential to make successful subunit vaccines from the many thousands of antigens that constitute this pathogen. Finally, the article from MacLennan also highlights that multiple antigens are likely to be targets of protective antibody and that this can be harnessed for vaccination. Nevertheless, this article also draws attention to the consequences of inappropriate levels of antibody responses that can turn a protective response to one that may actually be detrimental. Collectively, these three contributions highlight the challenges that we face to make effective vaccines to *Salmonella* infections.

This Research Topic has articles that consider the fundamental nature of *Salmonella* infections from the first principles of why one particular serovar causes one infection and a different one a distinct disease, all the way through to the nature of classical immunomodulation of the host through vaccination. Such a spectrum of offerings will help us better understand the nature of this pathogen and how it can control us and how we can control it.

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Same species, different diseases: how and why typhoidal and non-typhoidal *Salmonella enterica* serovars differ

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Human infections by the bacterial pathogen *Salmonella enterica* represent major disease burdens worldwide. This highly ubiquitous species consists of more than 2600 different serovars that can be divided into typhoidal and non-typhoidal *Salmonella* (NTS) serovars. Despite their genetic similarity, these two groups elicit very different diseases and distinct immune responses in humans. Comparative analyses of the genomes of multiple *Salmonella* serovars have begun to explain the basis of the variation in disease manifestations. Recent advances in modeling both enteric fever and intestinal gastroenteritis in mice will facilitate investigation into both the bacterial- and host-mediated mechanisms involved in salmonelloses. Understanding the genetic and molecular mechanisms responsible for differences in disease outcome will augment our understanding of *Salmonella* pathogenesis, host immunity, and the molecular basis of host specificity. This review outlines the differences in epidemiology, clinical manifestations, and the human immune response to typhoidal and NTS infections and summarizes the current thinking on why these differences might exist.

Keywords: *Salmonella enterica*, typhoid, enteric fever, NTS, salmonellosis, gastroenteritis

INTRODUCTION

Salmonella enterica is a highly diverse Gram negative bacterial species containing more than 2600 different serovars differentiated by their antigenic presentation. Various serovars are characterized by their host specificity or by the clinical syndrome they cause ranging from asymptomatic carriage to invasive systemic disease. Most *S. enterica* serovars associated with diseases in humans and other warm blooded animals belong to subspecies I consisting of both typhoidal and non-typhoidal serovars. Several excellent recent reviews have highlighted different aspects of invasive salmonellosis (De Jong et al., 2012; Feasey et al., 2012), discussed the mechanisms behind host restriction (Baumler and Fang, 2013), and detailed salmonelloses in immunocompromised individuals (Gordon, 2008; Maclennan, 2014). Here, we will discuss how typhoidal and non-typhoidal serovars differ in epidemiology, clinical manifestations, and the immune response they trigger in humans.

EPIDEMIOLOGY

While many non-typhoidal *Salmonella* (NTS) serovars such as Typhimurium and Enteritidis are generalist pathogens with broad host specificity, a few *S. enterica* serovars including Typhi, Sendai, and Paratyphi A, B, or C are highly adapted to the human host that is used as their exclusive reservoir. These specialist pathogens, collectively referred to as typhoidal *Salmonella* serovars, are the causative agents of enteric fever (also known as typhoid or paratyphoid fever if caused by serovar Typhi or Paratyphi, respectively). Enteric fever is an invasive, life-threatening, systemic disease with an estimated global annual burden of over 27 million cases, resulting in more than 200,000 deaths (Crump et al., 2004; Buckle et al., 2012). Enteric fever is endemic in the developing world in regions that lack clean water and adequate sanitation, facilitating the spread of these pathogens via the fecal-oral route. In recent years, for unknown reasons, the incidence of infections with serovar Paratyphi A is on the rise and in some regions of the globe, particularly in South–East Asia, this serovar is accountable for up to 50% of all enteric fever cases (Ochiai et al., 2005; Meltzer and Schwartz, 2010).

In contrast to typhoid fever which is common in the developing world, NTS salmonelloses occur worldwide. There are an estimated 93.8 million cases of gastroenteritis due to NTS infection each year, resulting in approximately 155,000 deaths (Majowicz et al., 2010). Despite global morbidity, mortality due to NTS infection is primarily restricted to the developing world. In addition to contaminated animal-derived food products such as poultry, eggs, and dairy products, NTS transmission can result from person to person contact or from contact with pets such as cats, dogs, rodents, reptiles, or amphibians (Hohmann, 2001; Mermin et al., 2004; Braden, 2006; Haeusler and Curtis, 2013). Another important source of infection is consumption of contaminated produce especially sprouts, tomatoes, fruits, peanuts, and spinach which have all been associated with recent outbreaks (Berger et al., 2009, 2010; Barton Behravesh et al., 2011; Cavallaro et al., 2011; Jackson et al., 2013; Bayer et al., 2014).

While normally NTS infections in humans induces gastroenteritis, in up to 5% of NTS cases, bacteria cause an invasive, extra-intestinal disease leading to bacteremia and focal

systemic infections, henceforth referred to as invasive NTS (iNTS; Mandal and Brennand, 1988). Interestingly, various NTS serovars (e.g., Typhimurium, Dublin, Choleraesuis, 9,12:l,v:−) tend to have more potential to cause extraintestinal infections than others. This implies there is a genetic basis for the emergence iNTS disease; however, these differences are still not understood (Wilkins and Roberts, 1988; Marzel et al., 2014). In Sub-Saharan Africa, iNTS is a major cause of bacteremia in adults and children, with an estimated annual incidence of 175–388 cases per 100,000 children and 2000–7500 cases per 100,000 HIV-infected adults. Especially *S.* Typhimurium sequence type (ST) 313 is associated with invasive disease. Startlingly, in 20–25% of cases, invasive infection results in the death of the patients. Other major risk factors for invasive disease in addition to HIV are co-infection with malaria and malnutrition (Feasey et al., 2012; Maclennan, 2014).

CLINICAL MANIFESTATIONS

Enteric fever caused by typhoidal serovars differs dramatically from the gastroenteritis normally associated with NTS. Infections caused by different typhoidal serovars (e.g., Typhi and Paratyphi A) cannot be distinguished by clinical presentation (Meltzer et al., 2005; Patel et al., 2010). The average incubation period for typhoidal serovars is 14 days with symptoms persisting for up to 3 weeks (Olsen et al., 2003; Wangdi et al., 2012). Patients most typically present with a gradual onset of sustained fever (39–40°C). Other frequent symptoms include chills, abdominal pain, hepatosplenomegaly, rash (rose spots), nausea, anorexia, diarrhea or constipation, headache, and a dry cough (Stuart and Pullen, 1946). In contrast to enteric fever, individuals infected with NTS have self-limiting, acute gastroenteritis and watery diarrhea. Nausea, vomiting, abdominal pain, and fever are also common symptoms (McGovern and Slavutin, 1979). With NTS infection, symptoms appear 6–12 h after the ingestion of the pathogen and clinical symptoms last less than 10 days (Glynn and Palmer, 1992). In the case of iNTS infections, which are often associated with patients with immunodeficiency, disease more closely resembles enteric fever in that patients often suffer from high fever, hepatosplenomegaly, and have respiratory complications with intestinal symptoms often being absent.

Both typhoidal and NTS serovars initially adhere to and invade the intestinal epithelium of the small intestine (Liu et al., 1988). Unlike NTS infection, infection by typhoidal serovars does not induce a high inflammatory response during the initial invasion of the intestinal mucosa (Sprinz et al., 1966; Kraus et al., 1999; Nguyen et al., 2004). Minimal intestinal inflammation during enteric fever is correlated with negligible neutrophil transmigration across the intestinal epithelium in contrast to massive neutrophil recruitment during intestinal inflammation caused by NTS serovars (McCormick et al., 1995). In immunocompetent patients, NTS gastroenteritis is self-limiting, with infection being confined to the terminal ileum and colon. In the case of typhoidal salmonellae, after passing the intestinal mucosa, bacteria gain access to underlying lymphoid tissues and multiply intracellularly within mononuclear phagocytes. Infection quickly becomes systemic with spreading of the pathogen from the intestine to the mesenteric lymph nodes, liver, spleen, bone marrow, and gallbladder. Secondary infection of typhoidal organisms to the small bowel can occur via secretion in the bile through the enterohepatic cycle (Gordon, 2008). The absence of robust intestinal inflammation and the lack of neutrophil transmigration are thought to facilitate the invasion of typhoidal serovars into the deeper tissues of the gut and its dissemination to systemic sites (House et al., 2001).

Interestingly, up to 10% of convalescing, untreated patients continue to shed *S.* Typhi in their stool for up to three months after infection (Parry et al., 2002). One to four percent of individuals infected with *S.* Typhi become asymptomatic, chronic carriers that continue to excrete 10^6–10^{10} *S.* Typhi bacteria per gram of feces for more than 12 months. The role of such chronic carriers in disease transmission was notoriously demonstrated by the case of Mary Mallon (Typhoid Mary). During her work at different households as a cook in the New York City area in the early 20th century, Mary Mallon infected between 26 and 54 people (Marr, 1999). Another example of an asymptomatic *S.* Typhi carrier was "Mr. N" who worked as a cowman and milker in South–East England and was responsible for a 207 case outbreak of typhoid fever, which peaked in 1899 but continued until 1909 (Mortimer, 1999). The suspected site of persistence of *S.* Typhi in carriers is the gallbladder and gallstones are thought to be an important risk factor for developing chronic carriage (Levine et al., 1982) as they are conducive for biofilm formation which protects bacteria from antimicrobial compounds and the host immune system. Long-term carriage of *S.* Paratyphi has received much less attention and is currently less characterized than *S.* Typhi, but a recent study in Nepal suggests a similar rate of persistence for serovars Typhi and Paratyphi A in endemic regions (Khatri et al., 2009; Dongol et al., 2012).

Long-term carriage of NTS has not been described. However, even though symptoms usually last only for a few days, adults excrete *Salmonella* on average for 1 month after infection and children under the age of 5 years shed bacteria in their feces for an average of 7 weeks (Buchwald and Blaser, 1984; Hohmann, 2001). Interestingly, several studies have shown that treatment with antibiotics can prolong shedding of NTS bacteria (Aserkoff and Bennett, 1969; Murase et al., 2000), although these findings are controversial (Dryden et al., 1996; Hohmann, 2001). In comparison to NTS serovars, the long-term persistence of typhoidal serovars in humans suggests an enhanced ability of these pathogens to evade the human immune system (Raffatellu et al., 2008b).

HUMAN IMMUNE RESPONSE

Infection in humans by NTS serovars induces a strong Th1 response with high levels of IFN-γ, IL-18, IL-12, IL-15, TNF-α, and IL-10 detected in serum from patients (Mizuno et al., 2003; Stoycheva and Murdjeva, 2005). Expression of several chemokines is also induced upon NTS infection, which leads to the recruitment and activation of macrophages and dendritic cells, and a significant influx of neutrophils into the intestinal lumen, which is a hallmark of NTS gastroenteritis. The fact that typhoidal serovars are not typically associated with acute diarrhea or a strong influx of neutrophils into the intestinal lumen (Sprinz et al., 1966; Kraus

et al., 1999; Nguyen et al., 2004) suggests that their initial interaction with the human gut mucosa is less inflammatory than that of NTS serovars.

Recent studies have shown that patients with inherited deficiency of the IL-12/IL-23 system (IL-12p40/IL-12Rβ1) are highly susceptible to NTS infections, but not to *S.* Typhi or *S.* Paratyphi infections, even though some of these patients live in endemic areas (MacLennan et al., 2004; Van de Vosse and Ottenhoff, 2006). These observations support the possibility that different inflammatory pathways may be involved in NTS vs. typhoidal infections including a distinct role for the IL-12 pathway. This idea is further supported by additional epidemiological observations indicating that invasive infections caused by NTS, but not by typhoidal serovars, are often associated with immunocompromised adults, in particular individuals infected with HIV (Gordon, 2008; MacLennan and Levine, 2013). This implies that certain immune responses, malfunctioning during HIV infection, are required for the immune defense against systemic infection of NTS, but not against typhoidal serovars.

The immune response to infection with typhoidal serovars is complex and involves both humoral and cell-mediated immune responses (Sztein, 2007). Clinical studies that examined the immune response of patients infected with *S.* Typhi showed a significant CD4 and CD8 T cell response to specific *S.* Typhi antigens during typhoid fever, with elevated levels of IFN-γ during the acute phase of the disease (Butler et al., 1993; Sheikh et al., 2011). Transcriptome analysis of peripheral blood mononuclear cells (PBMCs) from patients with acute typhoid fever also demonstrated up-regulation of the genes from the IFN-γ pathway compared to healthy individuals (Thompson et al., 2009). Induction of other cytokines in response to *S.* Typhi infection include IL-6 and IL-8 which are secreted into the serum during the acute phase of typhoid fever (Butler et al., 1993; Keuter et al., 1994; Gasem et al., 2003). PBMCs from immunized volunteers orally vaccinated with an attenuated *S.* Typhi vaccine secrete Th1 cytokines including IFN-γ, TNF-α, and IL-10, following sensitization with a number of *S.* Typhi antigens including flagella (Wahid et al., 2007). Collectively, these findings indicate that the human immune response to *S.* Typhi infection is predominantly Th1-associated.

Given that typhoidal serovars do not typically illicit septic shock, in contrast to many other Gram-negative pathogens that induce bacteremia and leukopenia (Pohan, 2004; Tsolis et al., 2008; Gal-Mor et al., 2012), suggests a restrained immune response mediated by these pathogens in the human host. This view is consistent with the clinical observation that serum levels of pyrogenic cytokines IL-1β and TNF-α are relatively low in patients with typhoid fever compared to the levels found in patients with sepsis caused by other Gram-negative pathogens. In fact, IL-1β and TNF-α production by PBMCs has been shown to be suppressed during the acute phase of typhoid fever (Butler et al., 1978; Girardin et al., 1988; Keuter et al., 1994; Gasem et al., 2003).

Despite the increasing prevalence of *S.* Paratyphi A in endemic regions, the immune response to *S.* Paratyphi infection is much less characterized than the one to *S.* Typhi. A recent study done in our group examined the circulating cytokine profile of healthy Israeli travelers that became infected with *S.* Paratyphi A during an outbreak in Nepal. Comparison of 16 cytokines demonstrated considerable (more than 10-fold) increase in the serum concentration of IFN-γ, but only a moderate elevation in the concentration of IL-6, IL-8, IL-10, and TNF-α between convalescence and the peak time of clinical presentation (Gal-Mor et al., 2012). These results suggest that the prominent IFN-γ and the moderate TNF-α, IL-6, and IL-8 responses are common to both typhoid and paratyphoid fever. Interestingly, no changes in IL-12 serum concentrations were detected during the acute phase of the disease (Gal-Mor et al., 2012), in contrast to its induction seen during gastroenteritis caused by NTS serovars (Stoycheva and Murdjeva, 2005).

CURRENT THERAPIES AND VACCINES

Antibiotic therapy can prolong the duration of excretion of NTS and therefore is only recommended for people with severe illness, invasive disease, or for certain risk groups including infants, the elderly, and immunocompromised individuals. Enteric fever, on the other hand is always immediately treated with antibiotics. In the 1990s, physicians moved away the first-line antibiotics chloramphenicol, ampicillin, and cotrimoxazole due to widespread resistance amongst *S. enterica* serovars. Since then, fluoroquinolones (like ciprofloxacin) have been the primary treatment for salmonelloses, as this class of drug is particularly effective against intracellular Gram-negative bacteria. While there is increasing resistance to fluoroquinolones, new fluoroquinolones like gatifloxacin hold promise. Third generation cephalosporins are often the second-line treatment to treat salmonelloses. In addition, azithromycin is relatively new drug with activity against both nalidixic acid resistant and multidrug resistant (MDR) strains (Hohmann, 2001; Arjyal and Pandit, 2008).

Multidrug-resistance is an increasing problem in *S. enterica* serovars. Resistance to multiple antibiotics is especially common in serovars Typhimurium and Newport and multidrug-resistant strains are also linked to more severe disease outcome (Krueger et al., 2014). Notably, many strains of *S.* Typhimurium Definitive Type (DT) 104, which have caused multiple outbreaks since the 1990s, are resistant to ampicillin, chloramphenicol, streptomycin, sulphonamides, and tetracycline (Mather et al., 2013). Moreover, new resistant strains of *S. enterica* are continuously emerging worldwide. For example, an MDR strain of serovar Infantis now accounts for up to 35% of the NTS infections in Israel (Gal-Mor et al., 2010; Aviv et al., 2014). Additional examples are the emergence of resistant strains of serovars Virchow (Weill et al., 2004) and Heidelberg (Dutil et al., 2010). Similarly, many iNTS strains are resistant against ampicillin, chloramphenicol, kanamycin, streptomycin, trimethoprim, and cotrimoxazole (Gordon, 2008; Kingsley et al., 2009; Msefula et al., 2012). Therefore, there is a high need to (i) prevent further resistance development through the prudent use of antibiotics, (ii) improve measures that prevent spread of MDR strains, and (iii) discover new therapies for salmonelloses. Interestingly, the re-emergence of chloramphenicol sensitive strains in areas where resistance was previously prevalent suggests that cycling or rotation of antibiotics could also

be an effective strategy to deal with antibiotic resistance, rendering older antibiotics useful once again (Abel Zur Wiesch et al., 2014).

Three types of vaccines against *S.* Typhi are currently commercially available, but unfortunately, there is still not a single licensed vaccine available against *S.* Paratyphi A, with very little, if any, cross-protection provided by the available *S.* Typhi vaccines. Vaccination strategies against typhoid fever including a description of ongoing trials were recently reviewed in detail (Waddington et al., 2014). The currently licensed *S.* Typhi vaccines include (i) a killed whole cell parenteral vaccine (Engels et al., 1998), (ii) a live attenuated oral vaccine, designated Ty21a (Germanier and Fuer, 1975) and, (iii) a Vi polysaccharide capsule-based vaccine (Tacket et al., 1986). There are vaccines against NTS serovars Enteritidis and Typhimurium which are effective in poultry (Desin et al., 2013). However, there are no vaccines available for NTS in humans or other animal reservoirs such as cattle or pigs. This represents a significant limitation in the existing prevention strategies. Understanding the host specificity determinants of *S. enterica* serovars will aid in future therapeutic and vaccine development.

WHY DO TYPHOIDAL AND NTS SEROVARS ELICIT SUCH DIFFERENT HOST IMMUNE RESPONSES?

How do pathogens so similar, belonging to the same subspecies (*S. enterica* ssp. I), with >96% DNA sequence identity between shared genes (McClelland et al., 2001) induce such different clinical manifestations and immune responses in humans? Despite significant advances in the field, this question is still far from being answered. Understanding the genetic and molecular mechanisms responsible for differences in disease outcome will aid in our understanding of *Salmonella* pathogenesis, host immunity, and the molecular basis of host specificity (**Table 1**).

In vitro tissue culture studies suggest that *S.* Typhi induces restrained inflammatory responses that do not trigger a pro-inflammatory response via TLR5. Similarly, polarized human colonic epithelial (T84) cells infected with *S.* Typhi induce significantly lower levels of the neutrophil chemoattractant IL-8 compared to *S.* Typhimurium infection (Raffatellu et al., 2005). Raffatellu et al. (2008b) have therefore postulated that *S.* Typhi expresses unique virulence factors that allow this pathogen to overcome the innate immune response in the intestinal mucosa resulting in the absence of neutrophil infiltration and inflammatory diarrhea. One of the current hypotheses in the field suggests that the polysaccharide capsular antigen Vi in *S.* Typhi enables this pathogen to resist phagocytosis and complement killing (Robbins and Robbins, 1984) and masks access to pattern recognition molecules, resulting in less IL-8 production (Raffatellu et al., 2005), limited neutrophil influx, and thereby reduced small bowel inflammation (Sharma and Qadri, 2004; Wilson et al., 2008). The role of the Vi antigen regulator TviA, and its putative contribution to *S.* Typhi's ability to evade the immune system have been recently reviewed (Wangdi et al., 2012). Nevertheless, since the Vi capsule is largely restricted to serovar Typhi and is absent from serovars Paratyphi A and Sendai, it cannot explain why the clinical manifestations of these other typhoidal serovars differ from that of NTS. Furthermore, the fact that Vi-negative mutants of *S.* Typhi are still able to cause a typhoid-like illness in human volunteers (Zhang et al., 2008), suggests that additional mechanisms are involved (**Figure 1**).

Of the approximately 4400 *S.* Typhi and *S.* Paratyphi A genes, about 200 are inactivated or functionally disrupted, while most of their homologs in *S.* Typhimurium are intact. Many of the degraded genes found in the genomes of the typhoid serovars are involved in motility and chemotaxis or encode for type 3 secretion system effectors, fimbriae, or adhesins that play a role in *Salmonella* pathogenicity (McClelland et al., 2004). Furthermore, *Salmonella* pathogenicity island (SPI)-7 (encoding the Vi antigen), SPI-15, SPI-17, and SPI-18 are present in the genome of *S.* Typhi, but not in the genome of *S.* Typhimurium, while SPI-14, present in *S.* Typhimurium, is absent from the genome of typhoidal serovars (Sabbagh et al., 2010). Therefore, it is highly possible that differences in virulence and colonization factor composition affect host–pathogen interactions and disease outcome in humans. This notion has recently been demonstrated by the expression of the *S.* Typhimurium effector, GtgE, in *S.* Typhi. When secreted into host cells, GtgE proteolytically degrades Rab29 and confers the ability of *S.* Typhi to survive and replicate within macrophages and in tissues from mice, a normally non-permissive host (Spano and Galan, 2012).

Recent evidence suggests that NTS serovars have evolved to flourish in the inflamed gut environment and use inflammation to outcompete microbiota (Stecher et al., 2007; Thiennimitr et al., 2011). It has been proposed that typhoidal strains may have lost this ability and therefore have evolved to not induce inflammation in the gut but rather thrive systemically. For example, a by-product of the acute intestinal inflammation triggered by *S.* Typhimurium and other NTS serovars is the generation of the terminal electron acceptors nitrate and tetrathionate in the lumen of the inflamed gut. These compounds can be used by *S.* Typhimurium and other NTS serovars to outcompete the fermenting gut microbes that are unable to utilize these electron acceptors (Winter et al., 2010). In another recent report, Nuccio and Baumler (2014) have identified a network of 469 genes involved in central anaerobic metabolic pathways that are intact in NTS, but are decayed in the genome of typhoid serovars. Some of these degraded genes include the ethanolamine utilization pathway (*eut* genes) as well as the vitamin B_{12} biosynthesis pathway (*cbi* and *cob* genes) required for ethanolamine utilization (Nuccio and Baumler, 2014). These pathways are hypothesized to enable NTS to utilize inflammation-derived nutrients to outcompete other gut microbes.

Collectively, a substantial degree of metabolic and virulence gene degradation exists in the genomes of typhoidal serovars which may explain the restricted host-tropism of these pathogens and may also provide at least a partial explanation as to why typhoidal and NTS-infections induce such different clinical presentations and immune responses in humans.

ANIMAL MODELS

ANIMAL MODELS OF NON-TYPHOIDAL SALMONELLOSES

There are several animal models used to model human gastroenteritis caused by NTS. The model which most resembles human disease is arguably infection of non-human primates (Kent et al.,

Table 1 | Summary of the differences between NTS and typhoidal serovars associated with disease in humans.

	NTS serovars	Typhoidal serovars
Serovars	Represented by the ubiquitous serovars Typhimurium and Enteritidis, but ∼1500 other serovars of *S. enterica* ssp. I are known	Typhi, Paratyphi, and Sendai
Host range	Broad	Human-restricted
Epidemiology	Worldwide	Endemic in developing countries especially Southeast Asia, Africa, and South America
Reservoirs	Farm animals, produce, pets	None, human to human transmission
Clinical manifestations	Self-limiting gastroenteritis in immunocompetent individuals (diarrhea, vomiting, cramps) In immunocompromised patients (including patients with inherited deficiency of the IL-12/IL-23 system and HIV), disease is associated with invasive extraintestinal infections	Invasive, systemic disease in immunocompetent individuals (fever, chills, abdominal pain, rash, nausea, anorexia, hepatosplenomegaly, diarrhea or constipation, headache, dry cough)
Disease course	Short incubation period (6–24 h) Brief duration of symptoms (less than 10 days) Long-term carriage has not been observed	Long incubation period (7–21 days) Extended duration of symptoms (up to 3 weeks) One to four percent of infected individuals become long-term (≥1 year) carriers
Human immune response	Robust intestinal inflammation, neutrophil recruitment, Th1 response	Minimal intestinal inflammation, leukopenia, Th1 response
Genetic basis of disease differences and host specificity	Low degree of genome degradation Able to use terminal electron acceptors for anaerobic respiration in the inflamed gut Unique virulence factors (e.g., fimbriae, SPI-14)	∼5% of the genome is degraded (e.g., inactivated metabolic and virulence factor genes) Unique virulence factors and pathogenicity islands (e.g., Vi antigen, SPIs 7, 15, 17, and 18)
Vaccination	No vaccine available for humans	(i) killed whole cell parenteral vaccine, (ii) live attenuated oral vaccine (Ty21a), (iii) Vi polysaccharide capsule-based vaccine
Animal models of human disease	Streptomycin-pretreated mice Calves Non-human primates	Mouse infection with *S.* Typhimurium *Tlr11*$^{-/-}$ mice Humanized mice

1966; Rout et al., 1974). Rhesus macacques are especially useful for investigating co-infection with simian immunodeficiency virus. For example, infection of SIV-infected macacques with *S.* Typhimurium results in a blunted immune response and invasive bacterial disease similar to what is seen in HIV-infected patients (Raffatellu et al., 2008a). Furthermore, this model is useful for testing the efficacy and safety of potential live *Salmonella* vaccines for HIV infected people (Ault et al., 2013). However, the use of primates is limited by ethical concerns, cost, and the inability for genetic manipulation. Infection of calves with *S.* Typhimurium results in similar pathology to humans. Furthermore, *S.* Typhimurium is a natural pathogen of cattle and beef is a common reservoir for human infection (Santos et al., 2001; Costa et al., 2012). Data from the calf model have provided valuable insights into host–*Salmonella* interaction. However, this model is also restricted by cost and the limited possibility for genetic manipulation of the host.

Due to the low cost, ease of housing/handling, and genetic manipulation possible, mouse models are the most widely used animal models to study bacterial disease. NTS infection of mice does not mimic gastroenteritis as seen in humans but results in a typhoid-like systemic disease. However, after pretreatment of mice with antibiotics such as streptomycin or kanamycin, *S.* Typhimurium can overcome the "colonization resistance" presented by the natural microbiota and thus efficiently colonize the cecum and colon. In the now widely used streptomycin pretreatment model, NTS infection has been shown to lead to overt inflammation characterized by transmural inflammation including epithelial destruction, infiltration of inflammatory cells into the mucosa, formation of crypt abscesses, submucosal edema, and hyperplasia (Barthel et al., 2003; Hapfelmeier and Hardt, 2005; Sekirov et al., 2008; Woo et al., 2008). This model is now being exploited by many research groups to dissect both the bacterial- and

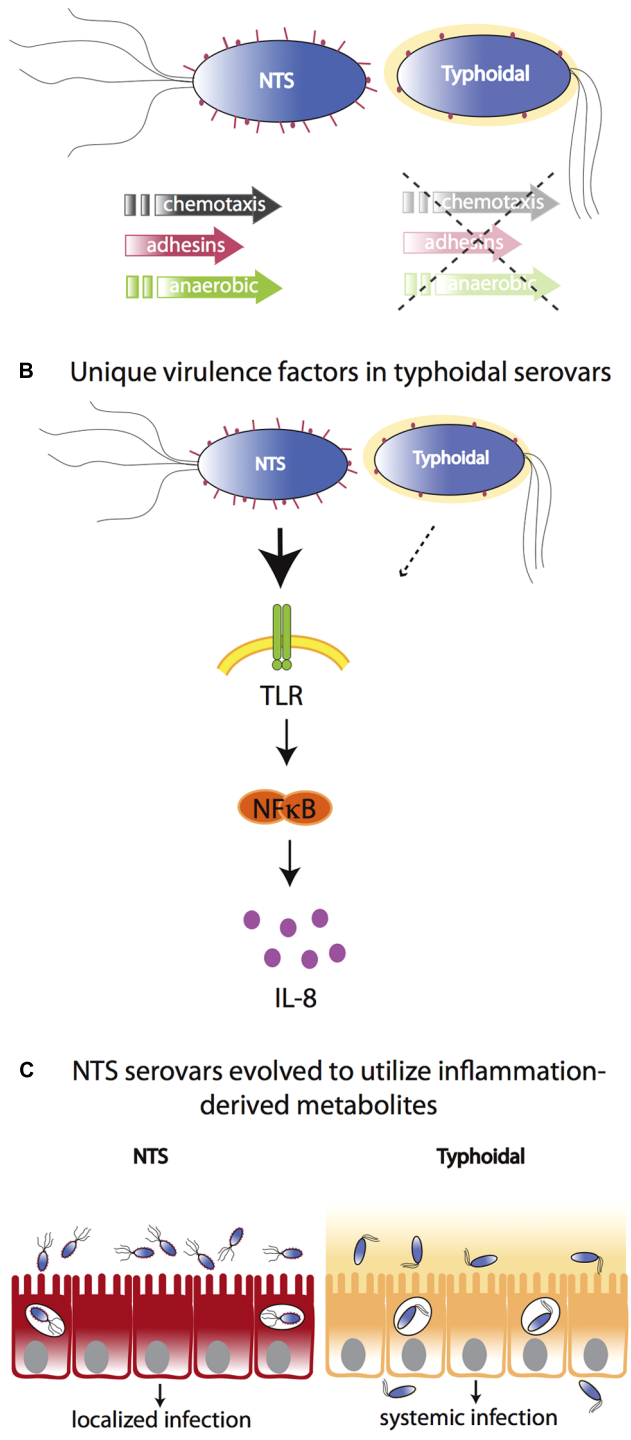

FIGURE 1 | Molecular bases for differences between typhoidal and NTS serovars. (A) Typhoidal serovars possess several inactive/degraded genes compared to NTS serovars such as genes for chemotaxis, adhesion, and anaerobic metabolism. **(B)** Both typhoidal and NTS serovars possess unique virulence factors. For example, some *S.* Typhi strains express Vi *(Continued)*

FIGURE 1 | Continued
capsule that reduces TLR-dependent IL-8 production in the intestinal mucosa. However, while the Vi capsule plays a role in typhoid fever manifestation, it is not necessary as it is absent from other typhoidal serovars and Vi-negative mutants of *S.* Typhi are still able to cause a typhoid-like illness in humans. **(C)** In contrast to typhoidal serovars, NTS cause severe intestinal inflammation. NTS serovars have evolved to utilize inflammation-derived metabolites (e.g., nitrate and tetrathionate), thereby enhancing their growth in the inflamed intestine. Typhoidal serovars have lost the ability to benefit from inflammation-derived metabolites and disseminate to systemic sites to a much greater extent.

host-mediated mechanisms involved in intestinal inflammation induction by NTS.

ANIMAL MODELS OF ENTERIC FEVER

S. Typhi, *S.* Paratyphi, and *S.* Sendai are human-restricted pathogens. Historically, attempts at eliciting enteric fever in animal models by infection with *S.* Typhi have proven to be rather inadequate. Chimpanzees infected with *S.* Typhi develop a mild disease that resembles enteric fever, but only when infected with a very high dose of 1×10^{11} CFU (Edsall et al., 1960). Another model for *S.* Typhi consists of inoculating mice intraperitoneally with *S.* Typhi suspended in hog gastric mucin (Pasetti et al., 2003). However, this model has not been found to correlate well with human enteric fever and with the expected attenuation of key *Salmonella* virulence regulators, such as PhoP (Baker et al., 1997).

Therefore until recently, due to the lack of suitable animal models, much of our understanding of enteric fever had been extrapolated from *S.* Typhimurium infection in mice. Mice infected with *S.* Typhimurium display minimal intestinal pathology but become systemically colonized as seen in humans with enteric fever. This model also allows for investigation of gallbladder colonization which is most likely the niche for chronic *S.* Typhi carriage in humans (Menendez et al., 2009; Gonzalez-Escobedo et al., 2013). Susceptible (*Slc11a1*$^{-/-}$, also known as *Nramp1*) mouse strains have been widely used but also resistant (*Slc11a1*$^{+/+}$) mice have proven useful. Mice with a wild-type *Slc11a1* gene (e.g., 129Sv, DBA) are relatively resistant to high doses of *S.* Typhimurium and have been particularly useful to investigate chronic infection, carriage (Lawley et al., 2006; Monack et al., 2004), and transmission (Lawley et al., 2008; Gopinath et al., 2012; Monack, 2012). In general, infection of mice with NTS has provided invaluable insight into the role of specific virulence factors in host invasion, dissemination, and transmission and although the murine inflammatory response to NTS in some ways resembles the human response to typhoidal serovars (Santos et al., 2001), conclusions from this model regarding the relevance to human typhoid disease must be carefully inferred.

In recent years, more sophisticated mouse models have been developed to study *S.* Typhi infection. Mathur et al. (2012) have shown that *Salmonella* flagellin is recognized in the mouse intestine by Toll-like receptor 11 (TLR11), which is absent from humans. *Tlr11* knockout mice are severely attenuated in innate epithelial responses to *S.* Typhi (and *S.* Typhimurium) and exhibit significant systemic infection following oral administration

(Mathur et al., 2012; Shi et al., 2012). It will be exciting to see if this model can also be used for infection with *S.* Paratyphi.

Another promising novel model is the use of humanized mice whereby immunodeficient mice (either *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ or NOD·Cg-*Prkdc*^{scid} *Il2rg*$^{-/-}$) lacking murine T, B, and NK cells are engrafted with human CD34+ hematopoietic stem cells (Shultz et al., 2007). These chimeric mice contain human immune cells including B cells, CD4^{+} and CD8^{+} T cells, NK cells, monocytes, and myeloid and plasmacytoid dendritic cells. Such humanized mice facilitate *S.* Typhi replication in the liver, spleen, and gallbladder and allow long-term persistence to be modeled (Song et al., 2010; Firoz Mian et al., 2011). In addition, infection results in a progressive, lethal infection within two to three days with inflammatory cytokine responses resembling human typhoid (Libby et al., 2010). These models suggest that the presence of human immune cells is prerequisite for systemic infection and *in vivo* replication of *S.* Typhi in the mouse. Although these humanized mice have proven informative to the study of *S.* Typhi infection, they are expensive and labor-intensive models and (so far) not widely used. Another limitation of such models is that they are subject to considerable inconsistency as a result of the genetic heterogeneity of donors and the variable degree of engraftment (Libby et al., 2010; Mian et al., 2011).

PERSPECTIVES

In-depth comparative analyses of the genomes of *Salmonella* serovars have begun to explain the basis for the variation seen in disease manifestations; however, this is still far from being fully understood. An interesting question in this regard is whether there is a genetic basis for the emergence of iNTS strains and why some NTS serovars (e.g., Typhimurium, Dublin, Choleraesuis, Schwarzengrund) tend to cause more invasive disease than others. In addition, the mechanisms by which co-infections (e.g., with *Plasmodium falciparum*, HIV) contribute to the increased risk of iNTS bacteremia must be further investigated. From the perspective of the host response, one unanswered question is whether there are unique immune responses to different typhoidal strains (e.g., Typhi vs. Paratyphi). And lastly, a fast-developing area of research that has already had implications on our understanding of salmonelloses is that of the role of the microbiota in disease outcome (see review by Santos in this issue). In the case of gastrointestinal pathogens, the influence of the host microbiota on pathogenesis, host immunity, and disease progression can no longer be overlooked.

Exploitation of the recent advances in modeling typhoid and NTS infection in mice is likely to provide novel insights into how these serovars are able to cause such different diseases. Opportunities remain, however, in the development of "next generation" humanized mouse models with enhanced human cell engraftment and function. These models hold much promise as they allow one to study the pathogenesis of human-restricted serovars, as well as to test the efficacy of therapeutic agents and experimental vaccines. Understanding the genetic and molecular mechanisms responsible for differences in disease outcome will aid in our understanding of *Salmonella* pathogenesis, host immunity, and the molecular basis of host specificity. Together, this information may be applied to control *Salmonella* infection, with specific determinants being targeted for therapeutic and vaccine development.

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Salmonella enterica in the chicken: how it has helped our understanding of immunology in a non-biomedical model species

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Salmonella infection of the chicken is important both as a source of foodborne human salmonellosis and as a source of disease in the chicken itself. Vaccination and other control strategies require an understanding of the immune response and as such have been important in understanding both mucosal immunity and more generally the response to bacterial infection. In this review, we discuss the contribution the study of avian salmonellosis has made to understanding innate immunity including the function of phagocytic cells, pattern recognition receptors, and defensins. The mucosal response to *Salmonella* infection and its regulation and the contribution this makes in protection against infection and persistence within the gut and future directions in better understanding the role of T_H17 and Tregs in this response. Finally, we discuss the role of the immune system and its modulation in persistent infection and infection of the reproductive tract. We also outline key areas of research required to fully understand the interaction between the chicken immune system and *Salmonella* and how infection is maintained in the absence of substantive gastrointestinal disease.

Keywords: *Salmonella*, chickens, innate immunity, adaptive immune responses, immune regulation, heterophils, toll-like receptors, mucosal immune system

INTRODUCTION

Salmonella enterica has a close relationship with the chicken, as poultry meat and eggs are regarded as the most important source of human foodborne infection (1). Furthermore, host-adapted serovars of *Salmonella* are important worldwide pathogens of the chicken causing the fowl typhoid and pullorum disease (2). As a consequence, *S. enterica* is the most studied bacterial pathogen in the chicken, not as in the case of the mouse and other biomedical models to determine the mechanisms of infection and immunity related to human disease, but with a specific focus on its control in the poultry industry. As such the development of vaccines and potential immunotherapeutic agents and studies based on understanding the transmission and carriage of *Salmonella* have been critical to our understanding of the function of the avian immune system.

Avian salmonellosis can be broadly divided into two main types based on infection biology. The majority of broad-host range *S. enterica* serovars are capable of infecting the chicken, usually leading to a period of colonization of lower gastrointestinal tract. In some serovars, notably *S.* Typhimurium and *S.* Enteritidis, this may be accompanied by a low-level systemic infection that is resolved through cellular immunity within two-to-three weeks (3, 4). Colonization is usually accompanied by activation of inflammatory responses in the ileum and the two large blind caeca that branch off at the junction of the colon and ileum (5, 6). Although infection with these serovars can lead to systemic disease in chicks or immunocompromised animals, in healthy immunocompetent animals of a week of age or more, infection leads to little or no signs of disease. In contrast are the two adapted serovars *S.* Gallinarum, the cause of fowl typhoid, and *S.* Pullorum, the cause of Pullorum disease (2). These serovars lead to a systemic infection, often with high levels of morbidity and mortality (7) Unlike the broad-host range serovars invasion via the gut is not accompanied inflammation allowing the establishment of systemic infection while avoiding activation of immunity (6, 8, 9). This avoidance of innate activation has been termed "stealth infection" and is also employed by *Salmonella* Typhi in human beings (10). Colonization of the gut by avian-adapted serovars is also poor, largely as a consequence of "functional genomic shrinkage" with the loss of genes or accumulation of pseudogenes leading to a reduced metabolic capacity forcing them into a systemic intracellular lifestyle (11). As in mammalian models of infection, *Salmonella* invade and persist within macrophages and dendritic cells, and, as in mice, the progression of infection is to a large extent dependent on the susceptibility of the animal (9). In experimental fowl typhoid in a susceptible chicken, infection rapidly becomes disseminated leading to septicemia (5). In resistant animals, infection is better controlled by macrophages and eventually cleared via adaptive responses. *S.* Pullorum is generally a less virulent pathogen of the chicken, but can lead to a persistent systemic infection or carrier state that can in turn lead to infection of the mature reproductive tract of the hen (12). The stages of infection in avian salmonellosis and interactions with the immune systems are summarized in **Figure 1**.

The diversity of interactions with the host by *S. enterica* in the chicken, in both in terms of the tissues and cell types involved and the steps taken by the bacterium to avoid and manipulate the

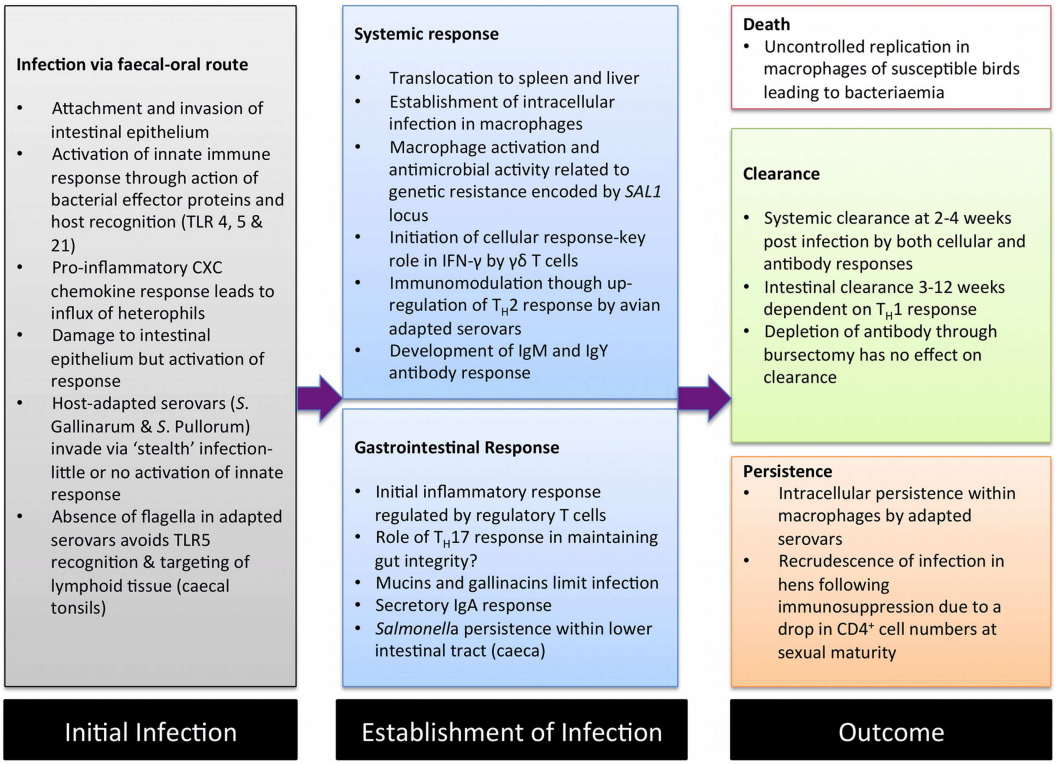

FIGURE 1 | A summary of the major interactions between *Salmonella enterica* and the chicken immune system. During avian salmonellosis initial interactions between pathogen and host innate immunity occur in the intestinal epithelium. Progression of infection and the related immune response is related to the infecting serovar or strain and to the host-genetic background. *Salmonella* is frequently invasive in chickens leading to both systemic and mucosal responses. Typically, in resistant animals systemic infection is transient and cleared by the adaptive immune response. However, in susceptible animals where macrophages fail to limit infection, a disseminated infection resulting in death can occur. Clearance from the intestinal tract may take a number of months and is associated with cellular responses. Systemic persistence leading to a carrier state may occur, in particular with *S.* Pullorum with bacteria persisting in low numbers for the lifetime of the bird.

immune system has revealed many similarities between the mammalian and avian systems that broadly function in the same way when challenged by *Salmonella*, yet there are a number of, sometime subtle, differences that reflect 200 million years of divergent evolution.

MAJOR DIFFERENCES BETWEEN THE AVIAN AND MAMMALIAN IMMUNE SYSTEMS – A BRIEF OVERVIEW OF A COMPACT IMMUNE SYSTEM

Functionally the immune system of the chicken behaves much the same way as that of mammals, perhaps reflecting a common ancestry. "Chickens are not feathered mice." a comment made by Jim Kaufman, a leader in the field of avian immunogenetics, clearly illustrates that there are key structural and functional differences found between the classes. Generally, the chicken immune system is more compact, with less polymorphism in its receptors and all but the IL-15 multigene family having fewer members than its murine equivalent. This is perhaps most clearly illustrated by the MHC Class I of the chicken, which has only two alleles with one dominantly expressed, leading to it being termed the "minimal essential MHC" (13). The chicken has only three immunoglobulin classes IgG (or IgY), IgM, and IgA and no IgG subclasses. Although the chicken TCR is considered to be less polymorphic there are two variants of αβ T-cells termed TCR1 and TCR2 along with γδ cells, which, interestingly, are found in greater numbers in the chicken. Toll-like receptors also have the same broad structure and function as mammals and recognize a similar array of ligands, though differences are found perhaps most markedly the absence of TLR9, which is replaced functionally by TLR21 (14), and the presence of TLR15, which has no known equivalent in mammalian systems (15). A comprehensive description of the avian immune system can be found in the recently published 2nd edition of 'Avian Immunology' (16).

INTERACTION WITH THE INNATE IMMUNE SYSTEM – INFORMING PHAGOCYTE FUNCTION, INFLAMMATION, AND TOLL-LIKE RECEPTORS

Salmonella usually infects chickens via the fecal–oral route with spread from the intestinal tract primarily at the distal ileum and caeca of the bird (1). Invasion is an inflammatory process leading to expression of proinflammatory cytokines and the chemokines CXCLi1 and CXCLi2, considered the equivalent of mammalian IL-8 (5, 6, 17, 18). This in turn leads to an influx of heterophils and monocytic phagocytes to the gut resulting in inflammation

and damage to the gut including fusion and flattening of the villi. Despite this enteropathogenic response, diarrheoa rarely occurs. While the bacterium itself induces cellular changes and inflammation through secreted effectors via its SPI1 Type III secretion system, recognition of flagellin through TLR5 appears to be the key event in the process (19). This is well illustrated by the fact that the non-flagellate avian-adapted serovars cause little inflammation during epithelial invasion *in vitro* or *in vivo* (9, 20), and that mutations in the flagellin gene of *Salmonella* Typhimurium lead to a more rapid invasion with lower initial levels of inflammatory signal (9, 19, 20). Indeed, this may be an evolutionary feature of adaptation to the avian host.

The consequence of activation of innate immunity is an influx of heterophils, the avian polymorphonuclear cell, and macrophages to the intestine. While these can lead to inflammatory damage, they also largely limit invasive disease. Our understanding of the biology and function of heterophils is almost entirely based on *Salmonella* infection studies. Depletion of heterophils changes *S.* Enteritidis from a gastrointestinal infection to a systemic infection illustrating their critical role in early immunity (21). Heterophils possess an array of TLRs (22), are efficient phagocytes, and can produce extracellular traps to facilitate this process (23). Unlike mammalian neutrophils, heterophils rely more on antimicrobial peptides as a bacterial killing mechanism (24) and although they produce nitric oxide and oxidative responses to *Salmonella* they lack the myeloperoxidase pathway (25). The study of the interaction of *Salmonella* with primary cultures of heterophils along with primary and continuous macrophage lines has been critical in our understanding of pattern recognition receptors in the chicken, including TLR5 as described above. Perhaps this is most clearly seen for TLR4 where variation in macrophage responses to *S.* Typhimurium challenge has identified both differences in levels of TLR4 expression and polymorphism in the receptor sequences between chicken lines. This suggests that responsiveness to LPS in chicken, which is frequently much lower than in mammals, is governed by variation in both levels of expression of the receptor and in the structure of the receptor itself (26, 27). Chicken TLR21 has no mammalian equivalent, though functionally it mirrors mammalian TLR9 in recognition of unmethylated (or CpG) DNA sequences. Much of our understanding of the response to CpG motifs has come through attempts to develop these sequences as immunostimulatory molecules or as vaccine adjuvant components to help control *Salmonella* (28, 29), although identification of the role of TLR21 was also founded in understanding the response to *Campylobacter jejuni* (14).

Macrophages differ little in structure or function to mammals, displaying a range of TLRs, expression of MHC Class II and phagocytic and antimicrobial activity. It is not yet understood whether avian macrophages have M1 or M2 phenotypes. The interaction with macrophages and dendritic cells and *Salmonella* is a key stage in the progression of systemic infection in particular. We have previously reviewed this in some detail (9), so will only briefly cover the essential points here. The use of inbred chicken models has identified the genetic locus *SAL1* that displays a phenotype of resistance to systemic salmonellosis (30). Macrophages derived from such birds shown enhanced oxidative killing and more rapid expression of key inflammatory and T_H1-assocated cytokines (31, 32). Fine mapping of this resistance locus has identified Akt1, a protein kinase, and Siva, a CD27-binding protein as functional candidates for the *SAL1* locus (33). A number of chicken macrophage-like cell lines are available and these have been utilized extensively to understand the interactions between *Salmonella* and this cell type in terms of cytokine response, the role of the bacterial SPI2 type III secretion system in intracellular survival and antimicrobial response to a range of serovars and have largely shown a common biology between mammalian and avian species (34–40).

As mentioned previously antimicrobial peptides play a key role in protection against avian salmonellosis. β-Defensins termed gallinacins in the chicken are produced by a range of cells and tissues in response to *Salmonella* infection or vaccination including, but not restricted to gallinacins 2–5 and 7 in gut epithelium (41–43). Gallinacins are also expressed during reproductive tract infection as described below. Like their mammalian equivalents gallinacins are cysteine-rich antimicrobials that have been shown to be active against a range of Gram negative and Gram positive bacterial species and have been considered as potential therapeutics in human medicine (44). Cathelicidins, also termed fowlcidins in the chicken, have also been described, but their role in salmonellosis is not known (44, 45). Other innate factors including increased expression of mucins, and in particular the gel-forming mucins (Muc2, Muc5ac, Muc5b, and Muc6), are likely to play a role in maintaining the epithelial barrier and limiting infection. Purified chicken mucin has been shown to have activity against *Campylobacter* (46), and work is ongoing in out laboratory to determine its role in enteric infections.

THE ADAPTIVE RESPONSE TO INFECTION AND THE SUCCESS OF VACCINATION

The success of vaccination programs such as those employed in the UK to reduce the burden of foodborne salmonellosis through control in egg and latterly poultry meat production is a clear indicator that protective adaptive immune responses can be elicited in the chicken (47). Infection with *Salmonella* elicits both antibody and cellular responses that can be detected from around a week post-infection. Clearance of both *S.* Enteritidis and the attenuated *S.* Gallinarum 9R vaccine strain from the spleen and liver is at around 2–3 weeks post-infection which coincides with high levels of interferon-γ expression and also production of IgM and IgG antibodies (5, 7, 48, 49). Preliminary adoptive transfer experiments have shown partial protection to systemic infection can be achieved by transfer of T lymphocytes (9).

In contrast, clearance from the intestinal tract is a much slower process. *Salmonella* infection leads to production of secretory IgA in the gut but any protective role is unclear as studies employing bursectomised (B lymphocyte-free) chickens give differing results dependent on the method employed. Both clearance and protection to re-challenge with *Salmonella* are reduced when hormonal or cyclophosphamide are used to deplete the Bursa of Fabricius (50, 51), whereas surgical bursectomy *in ovo* has no effect on the clearance of *Salmonella* or protection to re-challenge (52). Whilst the latter study suggests antibody is not required for clearance, the success of inactivated vaccines in *Salmonella* control in the chicken does suggest it plays an important role. However a number

of studies have shown that challenge elicits a strong Th1 response and that cellular immunity is more important in the chicken and clearance is dependent on age and cellular development. What we do not yet know is which effector mechanisms are employed in clearance. We do have some understanding of how the cellular response is activated. γδ-T lymphocytes are found in greater numbers in the chicken gut than mammalian systems and these cells play a key role in activation of adaptive response in the caeca and ileum. *Salmonella* challenge results in an influx of γδ lymphocytes and expression of IFN-γ, IL-12, and IL-18 leading to activation of T_H1 responses (53, 54). The γδ lymphocyte population has a heterogenous structure and phenotype in the chicken, with association of subsets with particular tissues (55). In the caeca, the $CD8^+\alpha\alpha^+$ γδ population is thought to be the main activator of the adaptive response (56).

MUCOSAL RESPONSES AND THE ROLE OF AND Tregs AND T_H17 CELLS

Given the importance of T_H17 cells in the mucosal inflammatory response, and as sentinels in the intestinal epithelium in mammals, there has been little focus on their role in avian salmonellosis. Furthermore, our understanding of the regulation of inflammatory responses and the role of regulatory T-cells in maintaining gut integrity following inflammatory responses is also limited. T_H17 cytokines are elicited rapidly after infection in the bovine ligated ileal loop *Salmonella* infection model (57), probably through stimulation of non-specific T_H17 cells while *Salmonella*-specific T_H17 cells possibly recognizing flagellin following activation via TLR5-dependent pathways may also contribute to intestinal mucosal protection (58). In the chicken IL-17 expression is upregulated in the cecum, the main site of bacterial colonization, following *S.* Enteritidis challenge though as yet no functional rule has been ascribed (42). Currently, the role of IL-17 is best characterized during infection by species of the chicken intestinal apicomplexan protozoan *Eimeria* where IL-17 may play a role both in protection and pathology dependent on the *Eimeria* species and co-infection with other enteric pathogens such as *Clostridium perfringens* (59–62).

The fact that many *Salmonella* serovars persist within the chicken intestinal tract with little sign of gastrointestinal disease despite eliciting a considerable inflammatory response and that inflammatory responses to *Salmonella* are relatively short-lived (5) strongly suggests there is a degree of regulation of this response. Our recent work on invasive *Salmonella* Typhimurium ST313 in the chicken illustrates this clearly (63); there is an initial CXCLi1 and CXCLi2 response leading to intestinal damage at three days post-oral infection, but by seven days post-infection this response is lowered and inflammatory damage largely resolved despite bacterial persistence (63). Some years ago, we showed that the lowering of intestinal proinflammatory signals following colonization with *S.* Typhimurium corresponded to increased expression of TGF-β, suggesting that regulation of inflammation was taking place (5). More recently the expression of IL-10 has been shown in the cecal tonsils in birds infected with *S.* Enteritidis at 4 days post-infection but not following infection the non-inflammatory avian-adapted serovars. It would seem likely that regulation of inflammatory immune responses, presumably by regulatory T-cells, allow *Salmonella* to persist within the gut for a number of weeks without disease to the bird but that the initial inflammatory response is sufficient to help control invasion and elicit responses that lead to systemic and eventually clearance of gastrointestinal infection. Such a "tolerogenic" response would have little or no impact on the bird itself, but has public health consequences in allowing persistence for several weeks, particularly given broiler chickens are typically slaughtered at around 6 weeks of age.

Recently, $CD4^+CD25^+$ cells have been identified as the avian equivalent of the mammalian Tregs, though the chicken appears to lack an ortholog of FoxP3 that are a characteristic feature of mammalian Tregs. Chicken $CD4^+CD25^+$ cells produce both IL-10 and TGF-β family cytokines and suppress T-cell proliferation *in vitro*. Stimulation of $CD4^+CD25^+$ *in vitro* or *in vivo* with *Salmonella* LPS, or infection, increases suppressive active. Intriguingly, $CD4^+CD25^+$ have also been shown to traffic to the cecal tonsil, suggesting this lymphoid organ at the ileal–cecal junction may play a key role in regulating intestinal immunity. There is clearly considerable scope to improve our understanding of chicken Tregs including the interaction with the intestinal microbiota, enteric pathogens, and in homeostasis of the healthy gut. Therapeutic approaches to deplete Treg function and thereby reduce suppression of the response to *Salmonella* have been proposed to reduce the carriage of *Salmonella* or *Campylobacter*. However, such an approach may well be detrimental to the health and welfare of chickens, leading to dysregulation of regulation of responses to the intestinal microflora resulting in poor gut health. Such an approach could also lead to uncontrolled inflammatory responses to *Salmonella* or *Campylobacter* infection leading to intestinal damage and diarrhea.

IMMUNOMODULATION IN PERSISTENT INFECTIONS

A feature of avian salmonellosis is persistent infection or carrier state. Intestinal carriage may occur for several months following infection with broad-host range serovars such as *S.* Typhimurium and *S.* Enteritidis, whereas avian-adapted serovars, most notably *S.* Pullorum, may persist in low numbers within macrophages in the liver and spleen for the lifetime of the animal. This persistence is in the face of a substantial immune response requiring evasion or modulation of the response by the bacterium. As discussed above immune clearance in the chicken is likely to be centered on T_H1-based cellular responses so avoiding these responses is key to pathogen survival. *S.* Pullorum is protected from antibody responses due to its intracellular niche, yet infection is associated with production of high titer IgG responses (12). Using a comparative approach between *S.* Pullorum and its close relative *S.* Enteritidis, we were able to show that systemic clearance of the latter was associated with a cellular response (9). In contrast, *S.* Pullorum infection leads to increased expression of IL-4 but unlike *S.* Enteritidis little expression of IFN-γ. This bias toward a T_H2 response would allow *S.* Pullorum to establish an intracellular carrier state avoiding T_H1-mediated clearance.

The mechanisms that underlie persistence in the GI tract are harder to determine. While as discussed above, regulation of the inflammatory response may help the establishment of a persistent infection, there is usually immune clearance in the long term.

As with systemic infection, the level and length of intestinal colonization is influenced by the generic background of the host. A recent study using inbred White Leghorn chickens of Line 6_1 considered susceptible to *Salmonella* colonization and Line N considered resistant (4), used a genome-wide transcriptional approaches to look at variations in enterocyte gene expression in an established GI tract infection (64). Both lines showed evidence of down-regulation of T_H1 responses, little evidence of stimulation of the T_H17 pathway, and no difference in expression of regulatory cytokines including IL-10 and TGF-β. In contrast the 6_1 susceptible line showed enhanced expression of key T_H2 cytokines including IL-4 and IL-13. This supports the notion that immune clearance of avian salmonellosis in T_H1 dominated and that T_H2 responses are associated with carrier states. As indicated by the authors, this is parallel with the murine model of *S.* Typhimurium where persistence is favored in M2 macrophage phenotypes that are driven by T_H2 cytokine responses.

INFECTION AND THE IMMUNE RESPONSE IN THE REPRODUCTIVE TRACT

A unique feature of avian salmonellosis is the frequent infection of the female reproductive tract and transmission to eggs by *S.* Enteritidis and *S.* Pullorum (12, 65). The structure and function of the immune system of the avian reproductive has been recently reviewed, reflecting the considerable progress in our understanding of its structure and function made in the last few years (66). Infection by *Salmonella* or stimulation with LPS results in a local innate response and in particular secretion of gallinacins throughout the reproductive tract, but in particular the lower part of the oviduct and uterus (67–69). There is also an organized T lymphocyte structure in the developing tract and IL-4 expressed within the tract that can lead to specific IgA responses. Sexual maturity in the hen has a profound effect on both systemic and local lymphocyte populations with a temporary fall in circulating T lymphocytes and particular $CD4^+$ cells and a loss of lymphocytic structure in the reproductive tract (70). This results in increased susceptibility to *Salmonella* challenge and decreased efficacy of vaccination at the start of the egg-laying period.

CONCLUSION AND FUTURE DIRECTIONS

Avian immunology has advanced greatly in recent years with the advent of genomic and transcriptomic approaches overcoming many of the difficulties due to lack of reagents, transgenic animals, or differences in the immune system that prevent the use of techniques commonly used in human and murine immunology. As transgenic chickens are now becoming available, functional studies on knockout chickens will no doubt follow. Nowhere will these be more welcomed than in understanding mucosal immunity, the "business end" of the response to *Salmonella.* There are a number of key questions that still need to be fully answered:

1. What are the mechanisms that underlie persistence of *Salmonella* in the chicken gut?
2. What regulates the GI response to prevent excessive intestinal damage?
3. Which effector mechanisms are important in clearance?

In addition to these, there are a number of areas, not least the role of microbiota in the development and homeostasis of the chicken mucosal immune system that require much work to improve our understanding of fundamental processes and mechanisms. While the ultimate aim of the avian *Salmonella* immunologist is to develop and improve vaccination and other controls that reduce the burden of *Salmonella* in food production, a better understanding of how the chicken regulates its response is as important, as disruption of this may have implications for the health and welfare of the animal itself, something that is increasingly important to the consumer.

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Genetic dissection of the *Ity3* locus identifies a role for *Ncf2* co-expression modules and suggests *Selp* as a candidate gene underlying the *Ity3.2* locus

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Typhoid fever and salmonellosis, which are caused by *Salmonella typhi* and *typhimurium*, respectively, are responsible for significant morbidity and mortality in both developed and developing countries. We model typhoid fever using mice infected with *Salmonella typhimurium*, which results in a systemic disease, whereby the outcome of infection is variable in different inbred strains of mice. This model recapitulates several clinical aspects of the human disease and allows the study of the host response to *Salmonella typhimurium* infection *in vivo*. Previous work in our laboratory has identified three loci (*Ity*, *Ity2*, and *Ity3*) in the wild-derived MOLF/Ei mice influencing survival after infection with *Salmonella typhimurium*. Fine mapping of the *Ity3* locus indicated that two sub-loci contribute collectively to the susceptibility of B6.MOLF-*Ity/Ity3* congenic mice to *Salmonella* infection. In the current paper, we provided further evidence supporting a role for *Ncf2* (neutrophil cytosolic factor 2 a subunit of NADPH oxidase) as the gene underlying the *Ity3.1* sub-locus. Gene expression profiling indicated that the *Ity3.1* sub-locus defined a global gene expression signature with networks articulated around *Ncf2*. Furthermore, based on differential expression and complementation analysis using *Selp* (selectin-P) knock-out mice, *Selp* was identified as a strong candidate gene for the *Ity3.2* sub-locus.

Keywords: *Salmonella, Ity3,* innate immunity, murine model for typhoid, selectin P, *Ncf2*

INTRODUCTION

Salmonella enterica, an intracellular Gram-negative bacterium, is the causative agent for a wide spectrum of clinical diseases with manifestations ranging from asymptomatic carriers, self-limiting gastroenteritis to fatal systemic infection (1, 2). There are over 2500 *Salmonella* serovars, of which, some are host adapted such as serovar Typhi and Paratyphi in humans, while others, such as Typhimurium and Enteritidis, have a broad host range and are capable of infecting multiple organisms. In humans, *Salmonella typhi* causes a systemic disease, typhoid fever, which has a global health burden of 26.9 million cases and 200,000 deaths annually (3). In humans, *Salmonella typhimurium* is the causative agent of salmonellosis, a self-limiting gastroenteritis that results from the consumption of contaminated food or water. The emergence of multi-drug resistant strains of *Salmonella* in recent years highlights the need for a more comprehensive understanding of the pathogenesis of *Salmonella* infection and for the identification of novel drug targets for vaccines and therapeutics (4).

Salmonella is a natural pathogen of mice and infection with *Salmonella typhimurium* results in a typhoid-like systemic disease. This murine experimental model has been used to identify several genes and pathways involved in disease pathogenesis (5–9). As there is limited genetic variation within the classical inbred strains, the use of wild-derived strains of mice, such as MOLF/Ei contributes added genetic diversity and has allowed for the identification of novel genes that play an important role in innate immunity (10–13). Classical and wild-derived strains of mice exhibit a range of susceptibilities to *Salmonella* infection; for example, the C57BL/6J classical inbred strain are extremely susceptible to infection with *Salmonella typhimurium* due to a mutation in *Slc11a1* (solute carrier family 11 member 1), while the 129 sub-strains are highly resistant (14). The wild-derived mouse strain, MOLF/Ei is also susceptible to infection despite carrying functional copies of genes known to be important in *Salmonella* infection, such as *Slc11a1* and *Tlr4* (toll-like receptor 4) (6, 10).

In order to identify the genetic determinants involved in the susceptibility of MOLF/Ei mice to *Salmonella* infection, we have previously used linkage analysis in an F2 panel of (C57BL/6 × MOLF/Ei) mice to identify two loci linked to host defense against *Salmonella typhimurium*, *Ity2* (Immunity to Typhimurium locus 2) and *Ity3* (10, 12). The MOLF/Ei allele at the *Ity2* locus improves resistance to infection, whereas MOLF/Ei allele at the *Ity3* locus confers susceptibility (15). Validation and fine mapping of *Ity3* locus were done using congenic B6.MOLF-*Ity/Ity3* mice (12) and a panel of 12 sub-congenic mice (16). Using this approach, the *Ity3* locus was refined to a 24 Mb interval and was shown to carry two sub-loci, *Ity3.1* and *Ity3.2* that together contribute to increased susceptibility to infection (16). The *Ity3.1* sub-locus controls NADPH oxidase activity and is characterized by decreased reactive oxygen species (ROS) production, reduced

inflammatory cytokine response, and increased bacterial burden. The *Ity3.2* sub-locus is characterized by a hyper-responsive inflammatory cytokine phenotype after exposure to *Salmonella* (16). Sequencing, expression, and functional data support the candidacy of *Ncf2* (neutrophil cytosolic factor 2 a subunit of NADPH oxidase) as the gene underlying the *Ity3.1* sub-locus (13).

In the current study, we used global expression profiling to better understand the genetic networks that are being influenced by the *Ity3* sub-loci and to identify potential candidate genes for the *Ity3.2* sub-locus. We illustrate the impact of the *Ity3.1* sub-locus on cell death and cytoskeletal reorganization, hematopoiesis as well as propose the candidacy of *Selp* (selectin P) as one of the candidate genes underlying *Ity3.2* based on expression analysis, coding sequence polymorphism, and functional and allelic complementation studies.

MATERIALS AND METHODS

ETHICS STATEMENT

All animals were maintained at the Animal Care Facility of McGill University according to the guidelines of the Canadian Council on Animal Care (CCAC). The animal protocol for this study was approved by the McGill University Animal Care Committee.

ANIMALS

Classical inbred strain C57BL/6J and wild-derived MOLF/Ei mice were used to generate congenic, B6.MOLF-*Ity* and B6.MOLF-*Ity/Ity3* and sub-congenic mice as described previously (12, 16). The susceptible *Ity3* and resistant *Ity* mice, as well as the intermediate B6.MOLF-*Ity/Ity3.RecN* and B6.MOLF-*Ity/Ity3.RecG* mice were used for the microarray expression analysis, while the B6.MOLF-*Ity* and B6.MOLF-*Ity/Ity3*, crossed with B6.129S7-*Selp*^{tm1Bay}/J ordered from the Jackson Lab (Bar Harbor, ME, USA), were used for the complementation assay.

IN VIVO SALMONELLA INFECTION

Mice aged 7–12 weeks were infected with *Salmonella typhimurium* strain Keller as described previously (12, 16). Briefly, mice were inoculated with 0.2 ml of physiological saline containing 10^3 colony-forming units of bacteria through the caudal vein. The infectious dose was verified by serial dilutions on trypticase soy agar. Mice were either monitored for survival or euthanized at day 3 or day 5 post-infection for organ collection. The animals were monitored two to three times daily and mice showing body condition scoring <2.0 were used for clinical endpoint (17). Survival analysis was conducted using a Kaplan–Meier survival test.

MICROARRAY EXPRESSION ANALYSIS

RNA was extracted from the spleens of mice, which were collected before infection and at day 3 post-infection. The RNA extraction was carried out using TRIzol reagent (Invitrogen Canada, Inc., Burlington, ON, Canada). Three age-matched male mice were used per group. The concentration of RNA was determined using a NanoDrop spectrophotometer (Thermo-Fisher Scientific, Waltham, MA, USA). All hybridization and scanning of mice microarrays were carried out at the McGill University and Genome Quebec Innovation Centre, using the Illumina BeadArray technology (Illumnia Inc., San Diego, CA, USA). The expression data were analyzed using FlexArray and normalized using a Lumi algorithm (Illumnia). Following the normalization, two approaches were used to generate a list of genes differentially expressed across the various strains. First, a Cyber *t*-test (Baladi and Long) (18) was used to generate a list of genes differentially expressed during infection, by comparing the fold change in expression of each gene between infected and uninfected samples for each strain. These gene lists were further refined using the Benjamini Hochberg false discovery rate algorithm. Genes with an FDR *p*-value <0.1 and a fold change of >2 were used to generate a final list of genes, which represented genes differentially regulated in each strain upon infection.

A second approach was used to generate a list of genes that were differentially expressed at each time point, as compared to the control *Ity* strain. This was done by comparing the expression of genes in each strain to the control *Ity* strain at both day 0 and day 3 post-infection. The gene lists were further refined using the Benjamini Hochberg false discovery rate algorithm. Genes with an FDR *p*-value of <0.05 was used as a cut-off to characterize genes as significantly differentially regulated as compared to *Ity*. The gene lists generated using the two approaches were studied using a suite of online tools including DAVID (19), GeneGo (MetaCore, Thomson Reuters) and Gene Mania (20, 21).

SEQUENCING OF *Selp*

Sequencing was performed on PCR-amplified cDNA from *Ity* and *Ity3* congenics obtained by reverse transcription of Trizol spleen-extracted RNA to determine genetic variation between C57BL/6J and MOLF/Ei alleles of the *Selp* candidate gene. Sanger sequencing was completed at the McGill University and Génome Québec Innovation Centre.

ALLELIC COMPLEMENTATION ASSAY

In order to study the effect of a MOLF/Ei *Selp* allele on susceptibility to infection, we carried out a complementation cross. *Selp* knock-out mice, B6.129S2-*Selp*^{tm1Hyn}/J (*Selp*$^{-/-}$) were ordered from the Jackson Laboratories (Bar Harbor, ME, USA). These mice were on C57BL/6J background with a mutant *Slc11a1* allele. In order to correct for this, we crossed the *Selp*$^{-/-}$ mice to *Ity* as well as to the *Ity3* mice. Mice were genotyped for the *Selp*$^{-/-}$ allele, and mice carrying the MOLF/Ei *Slc11a1* allele along with the *Selp*$^{-/-}$ allele were inter-crossed to generate homozygous *Selp*$^{-/-}$ mice with a MOLF/Ei allele at the *Slc11a1* gene. Furthermore, these mice were crossed with *Ity* or *Ity3* mice to generate mice, which are homozygous wild-type at *Slc11a1*, but carry a *Selp* knock-out allele complemented by either a C57BL/6J or MOLF/Ei allele (Figure S1 in Supplementary Material).

BACTERIAL LOAD ENUMERATION

For bacterial burden quantification in the spleen and the liver, mice were euthanized using CO_2 and at the required day post-infection; both organs were removed aseptically, weighed and homogenized using a Polytron (Kinematica, Bohemia, NY, USA). The resulting homogenate was diluted in 0.9% saline and plated on tryptic soy agar to determine organ bacteria burden.

STATISTICAL ANALYSIS

Statistical analysis was performed using Graph Pad Prism 6 (GraphPad Software, San Diego, CA, USA). One-way ANOVA with Dunnet's multiple correction test was used to analyze the bacterial burden in the spleen. A corrected p-value <0.05 was used to establish significant differences.

RESULTS

Ity3 INFLUENCES THE EXPRESSION OF SPECIFIC GENES AND PATHWAYS DURING INFECTION

The *Ity3* locus is a complex QTL containing at least two sub-loci. We have previously studied the phenotypic impact of these two sub-loci (16), and in order to further characterize the genetic networks that are affected by the MOLF/Ei allele at the *Ity3* locus, we have used a microarray expression approach. Using this approach, we should be able to characterize and identify candidate genes within each sub-loci and identify pathways, which affect the host immune response to *Salmonella* infection. We studied the splenic global expression profile of infected and uninfected congenic resistant B6.MOLF-*Ity* (*Ity*), susceptible B6.MOLF-*Ity/Ity3* (*Ity3*), and *Ity3.RecG* and *Ity3.RecN* strains. These two sub-congenic strains were selected because they carry either the MOLF/Ei alleles at *Ity3.1* (*Ity3.RecG*) or at *Ity3.2* sub-locus (*Ity3.RecN*), which result in an intermediate survival phenotype after infection with *Salmonella typhimurium* (16). *Ity*, *Ity3*, *Ity3.RecN*, and *Ity3.RecG* mice show significant differences in spleen and liver bacterial burden by day 5 post-infection (16). To evaluate primary defects in gene expression rather than differences due to high bacterial load, we selected day 3 as the time point to be studied because there was no significant difference in the spleen bacterial burden among the four strains of mice (16). Therefore, any changes in gene expression will serve as an indicator for genes and pathways that are important in regulating the susceptibility to *Salmonella* infection and not a consequence of a difference in bacterial load within strains. Our aim was to identify transcriptional signatures common to both *Ity3* and *Ity3.RecG* to define pathways controlled by the *Ity3.1* locus and those similarly regulated by *Ity3* and *Ity3.RecN* to identify transcriptional networks controlled by the *Ity3.2* locus. We used two approaches to study the gene expression profiles (**Figure 1**).

As an initial approach, we studied the changes in splenic gene expression in each strain over time (Day 3–Day 0). Overall, 241 (*Ity*), 204 (*Ity3*), 218 (*Ity3.RecG*), and 201 (*Ity3.RecN*) genes were differentially expressed during infection as defined by a cut-off of a fold change >2 and a p-value <0.1 (Tables S1A–D in Supplementary Material; **Figure 1**). We identified 129 genes that were commonly and significantly regulated during infection in all four strains including a number of pro-inflammatory genes (Table S1E in Supplementary Material). The majority of these genes were chemokines, cytokines, and other immune related genes with a significant number of genes regulated by Type I and Type II IFN including several members of the *Gbp* family, *Stat1*, *Usp18* as well as others that are known to be involved in *Salmonella* infection (7, 15). These genes (Table S1E in Supplementary Material) define a transcriptional signature that is common during *Salmonella* infection and has been previously detected in different strains of mice during infection (15, 22). Additional strain-specific genes regulated during infection were detected only in *Ity* (40 genes), *Ity3* (20 genes), *Ity3.RecN* (62 genes), and *Ity3.RecG* (54 genes) (Tables S1F–I in Supplementary Material). A large number of differentially expressed genes specific to *Ity* mice (Table S1F in Supplementary Material) were up-regulated in granulocytes and/or macrophages including *S100a8* and *S100a9* that are of particular interest as they are involved with expression of inflammatory mediators, phagocytosis, oxidative burst as well as migration of neutrophils and monocytes to the site of infection (23). The gene *Clec7a* (*dectin 1*) was differentially regulated only in *Ity*. Recent work has linked dectin 1/Syk kinase signaling with autophagy-dependent maturation of phagosomes (24).

In *Ity3* mice, most genes that were differentially regulated were either expressed in macrophages or in megaerythrocyte precursors suggesting active extramedullary erythropoiesis in this strain (Table S1G in Supplementary Material). Interestingly, a large proportion (~40%) of genes specific to the strain *Ity3.RecG* are known to be down-regulated in B and T cells, as analyzed by BioGPS (25) (Table S1G in Supplementary Material). These data show the impact of *Ity3* on the cellular composition of the spleen and/or changes in gene expression in specific cellular populations during infection. Very few genes were similarly regulated between *Ity3* and the sub-congenic strains *Ity3.RecG* (4 genes), *Ity3.RecN* (7 genes), and *Ity3.RecN* and *Ity3.RecG* (4 genes) (Tables S1J–L in Supplementary Material).

We then used GeneGO (Thomson Reuters, NY, USA) to classify the genes differentially regulated in each strain (Tables S1A–D in Supplementary Material) into gene ontology (GO) molecular pathways, GO processes, pathways, and process networks, in order to identify the pathways differentially regulated in each strain during infection (**Figure 2**). The strain *Ity3.RecG* appeared to have fewer genes involved in various pathways and processes related to chemokine and cytokine activity and immune response as demonstrated by the lower $-\log_2$ (p-values). These results are consistent with previous observations of reduced inflammatory responses following *in vivo Salmonella* infection in *Ity3.RecG* mice (16).

Ity3.1 LOCUS INFLUENCES THE EXPRESSION OF GENES INVOLVED IN CELL-CYCLE REGULATION AND HEMATOPOIESIS

We have previously reported that *Ncf2* is a strong candidate for the *Ity3.1* locus (13). To further characterize the downstream impact of *Ity3.1* and *Ncf2* on gene expression, we analyzed the data by evaluating variation in gene expression between *Ity3*, *Ity3.RecG*, or *Ity3.RecN*, and the control strain *Ity* at day 0 and day 3 post-infection (**Figure 1B**). Interestingly, 12 genes located within the introgressed *Ity3* region had lower expression levels in *Ity3* and *Ity3.RecG*, compared to both *Ity* and *Ity3.RecN*. **Figure 3A** shows the box plot expression pattern of one of these genes and the list is provided in Table S2A in Supplementary Material. None of these genes were differentially regulated during infection in any of the sub-congenic strains. For some of these genes, the low levels of expression detected in mouse strains *Ity3* and *Ity3.RecG* appear to be a consequence of poor hybridization of MOLF/Ei cDNA to microarray probes as a result of high genomic variability between the MOLF/Ei and C57BL/6J strains (Figure S2 in Supplementary Material). The wild-derived inbred MOLF/Ei had been separated from the classical inbred trains by over 1 million year

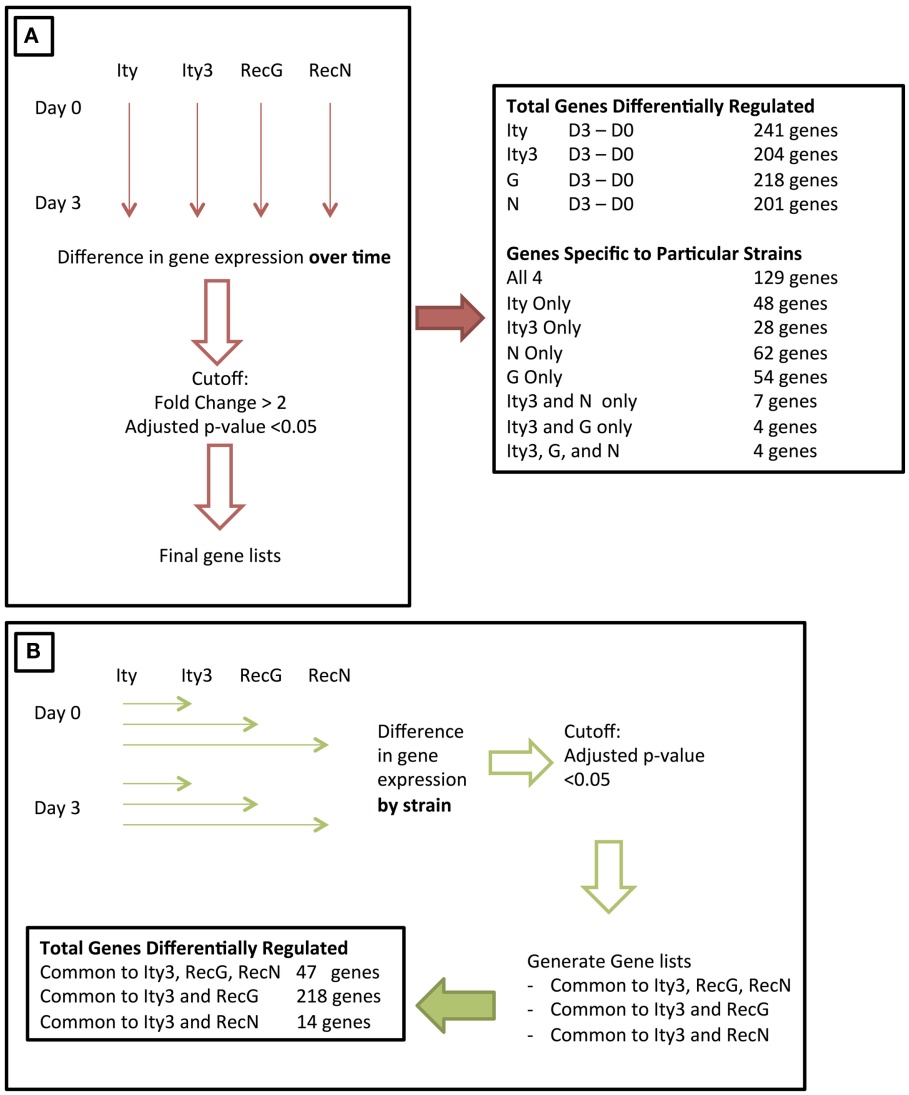

FIGURE 1 | Figure illustrating the two approaches used to study the changes in gene expression during infection of congenic and sub-congenic mice. **(A)** A representation of an initial approach used for studying the change in gene expression over time. The gene expression at day 3 post-infection was compared to day 0, and the final number of genes differentially regulated is stated on the right. **(B)** The second approach used, where gene expression in each strain was compared to the control *Ity* strain, at both day 0 and day 3. The final number of genes within each category is shown at the bottom of the panel.

of evolution, and as a result they have accumulated significant sequence variability, to the order of 1 SNP every 100 bp (26, 27).

A second set of genes showed similar patterns of up-regulation (**Figure 3B**; Table S2B in Supplementary Material) or down-regulation (**Figure 3C**; Table S2C in Supplementary Material) during infection in all strains, although the constitutive and induced expressions were similar in *Ity3* and *Ity3.RecG* but significantly different from *Ity* and *Ity3.RecN*. This expression pattern highlights the impact of the MOLF/Ei allele at the *Ity3* locus, as all the genes, which an expression pattern similar to **Figures 3B,C** carry a MOLF/Ei allele at the *Ity3.1* locus (16).

Another set of genes showed higher expression during infection only in strain *Ity3* and *Ity3.RecG* (**Figures 3D,E**; Tables S2D,E in Supplementary Material) as compared to *Ity*. In contrast to this grouping, the cluster of genes in **Figure 3F** exhibit lower expression levels in *Ity3* and *Ity3.RecG*. Collectively, these groups of genes in **Figures 3D–G** (Tables S2D–G in Supplementary Material) exhibit a pattern of expression that is similar between *Ity3* and *Ity3.RecG*, and the expression differences could not be attributed solely to the MOLF/Ei allele at chromosome 1, therefore, we can conclude that the expression differences are likely under the influence of the *Ity3.1* locus.

Functional annotation of genes, which have an expression pattern similar between *Ity3* and *Ity3.RecG* (Tables S2G–D in Supplementary Material) showed that a large percentage of the genes

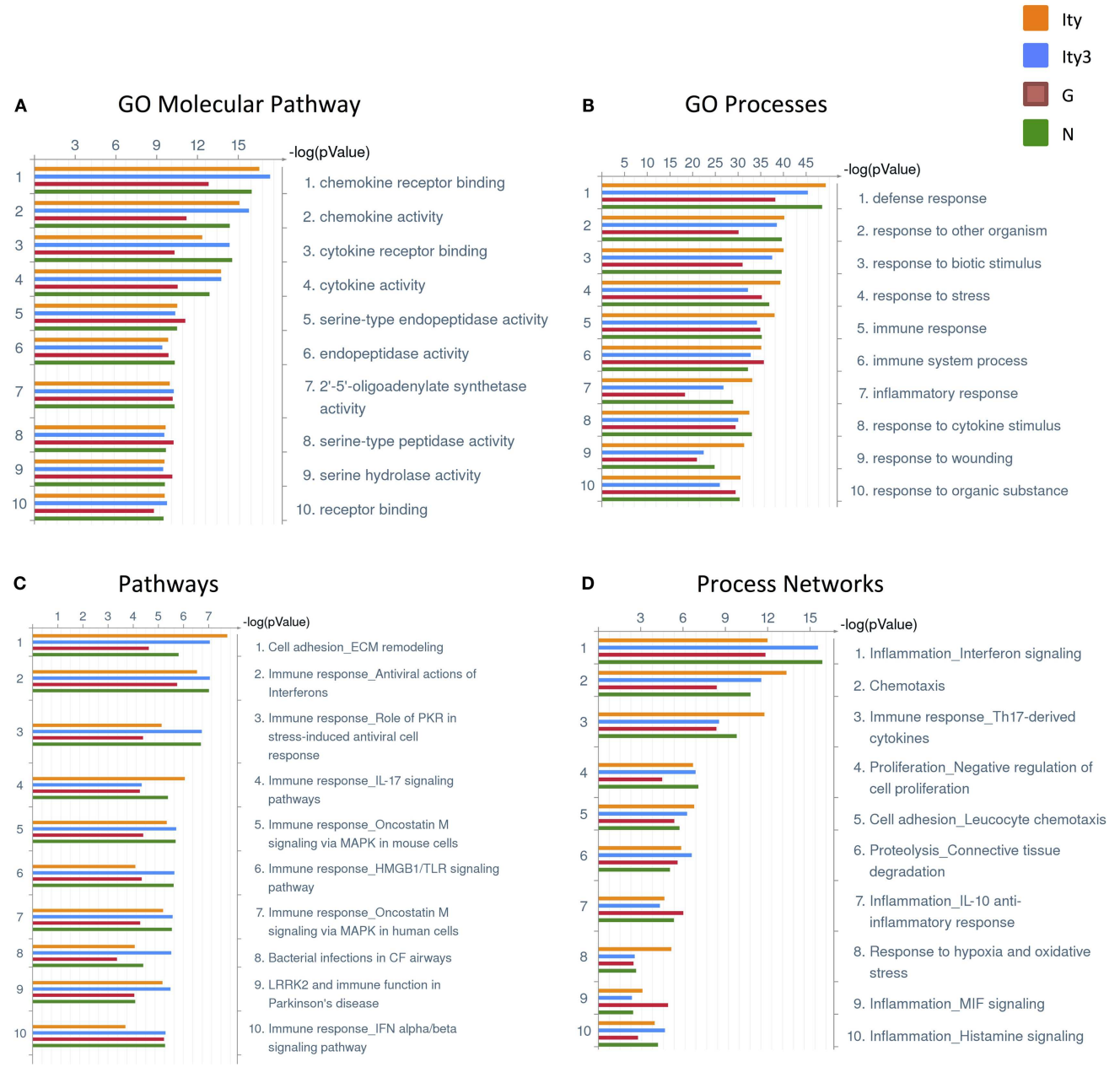

FIGURE 2 | Pathway and process analyses of genes differentially regulated in the spleen of *Ity, Ity3, Ity3.RecG,* and *Ity3.RecN* mice during infection. Gene ontology classifications generated by clustering the genes that are differentially regulated in each strain upon infection. **(A)** Gene ontology (GO) molecular pathways and **(B)** Gene ontology (GO) processes **(C)** pathways **(D)** Process networks that are enriched in the four mouse strains. The sub-congenic strain *Ity3.RecG* shows a lower −log(*p*-value) for chemokine and cytokine receptor activity within the GO molecular pathways as well as lower −log(*p*-values) for response to bacteria in the GO processes. This is consistent with previous data suggesting that the *Ity3.RecG* mice have a diminished inflammatory response following infection compared to parental strains (16).

in the list plays a role in cell cycle, DNA binding, cytoskeletal reorganization, and hemopoietic and lymphoid organ development (Table S3 in Supplementary Material) (19, 28). Another major category of genes such as *Ank1* and *Uros* are involved in heme metabolic process. These data are consistent with the observation that ROS control cell-cycle progression by influencing the presence and activity of cyclins and cyclin dependent kinases (29) and with a role for ROS in maturation and lifespan of erythroid cells (30, 31).

Ity3.1 AFFECTS A GROUP OF GENES THAT ARE CO-EXPRESSED WITH *Ncf2*

In order to define the gene expression profile of the susceptible strains, we identified genes that had a similar pattern of

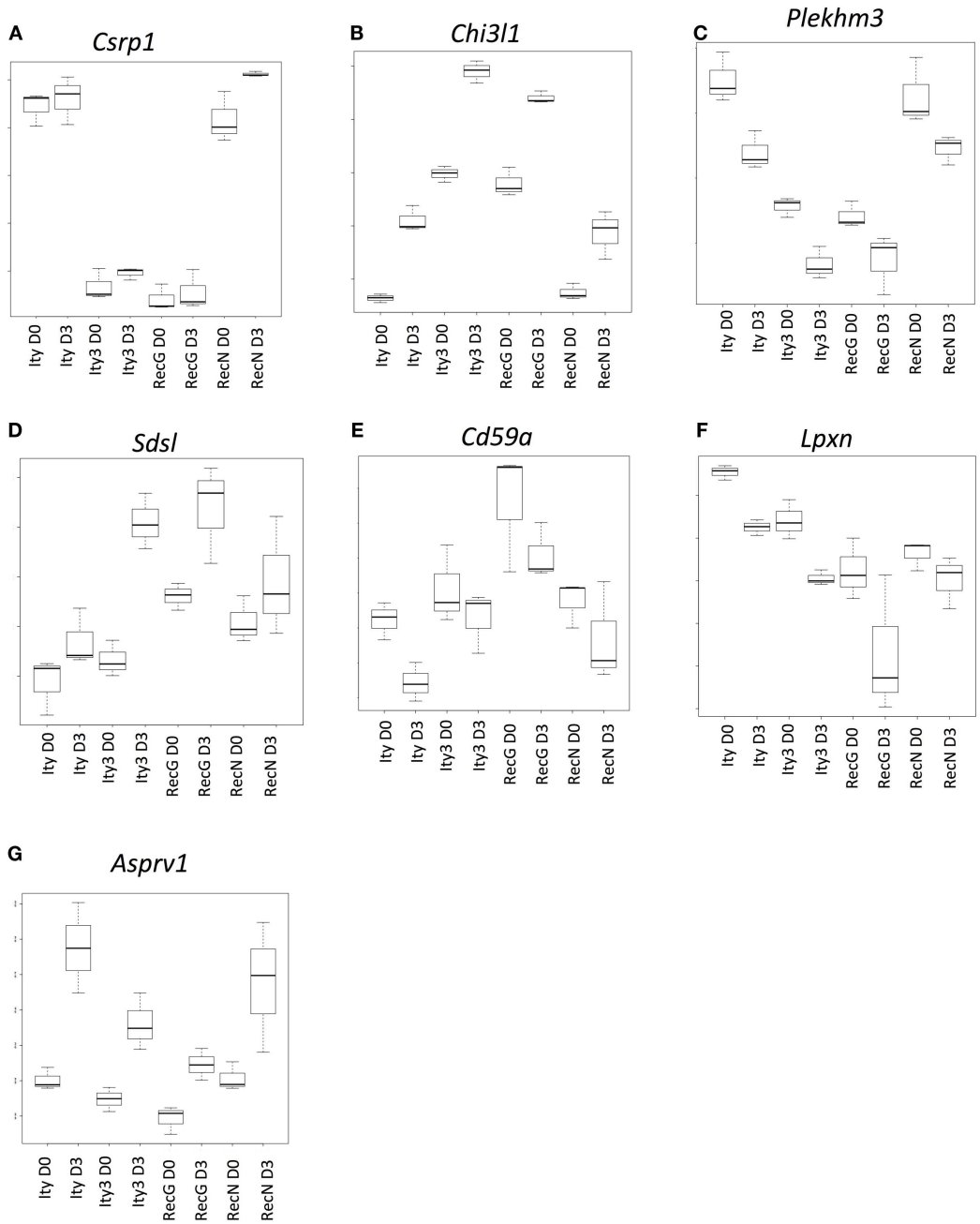

FIGURE 3 | Genes that are under the control of the *Ity3.1* locus. Sample box plots of the gene lists provided in Table S2 in Supplementary Material are shown. **(A)** Represents genes, which do not show any changes in expression during infection and show a similar expression pattern in *Ity3* and *Ity3.RecG*. **(B,C)** Represents a sample box plot of gene expression, which show a similar regulation pattern in *Ity3* and *Ity3.RecG*. **(D–G)** Also illustrate genes within Tables S2D,G in Supplementary Material, which show similar expression patterns in *Ity3* and *Ity3.RecG*, but different from *Ity* and *Ity3.RecN*. These genes are likely under the control of the *Ity3.1* locus. *Csrp1* (cysteine and glycine-rich protein 1), *Chi3l1* (chitinase-like 1), (*Plekhm3*) Pleckstrin homology domain containing, family M, member 3), *SdsI* (serine dehydratase-like), *Cd59a* (CD59a antigen), *Lpxn* (leupaxin), *Asprv1* (aspartic peptidase, retroviral-like 1).

expression in the susceptible strain, *Ity3* as well as the two subcongenic strains *Ity3.RecN* and *Ity3.RecG*. **Figures 4A,B** highlights two genes, *Tor3a* and *Fam20b* as examples of the expression pattern of the list of genes provided in Table S2H in Supplementary Material, which have a similar expression pattern in *Ity3*, *Ity3.RecN*, and *Ity3.RecG*. Only 7 of the 47 genes were within the *Ity3* interval, and almost all of them were within the genomic region common to *Ity3.RecN* and *Ity3.RecG*. This gene list was classified within

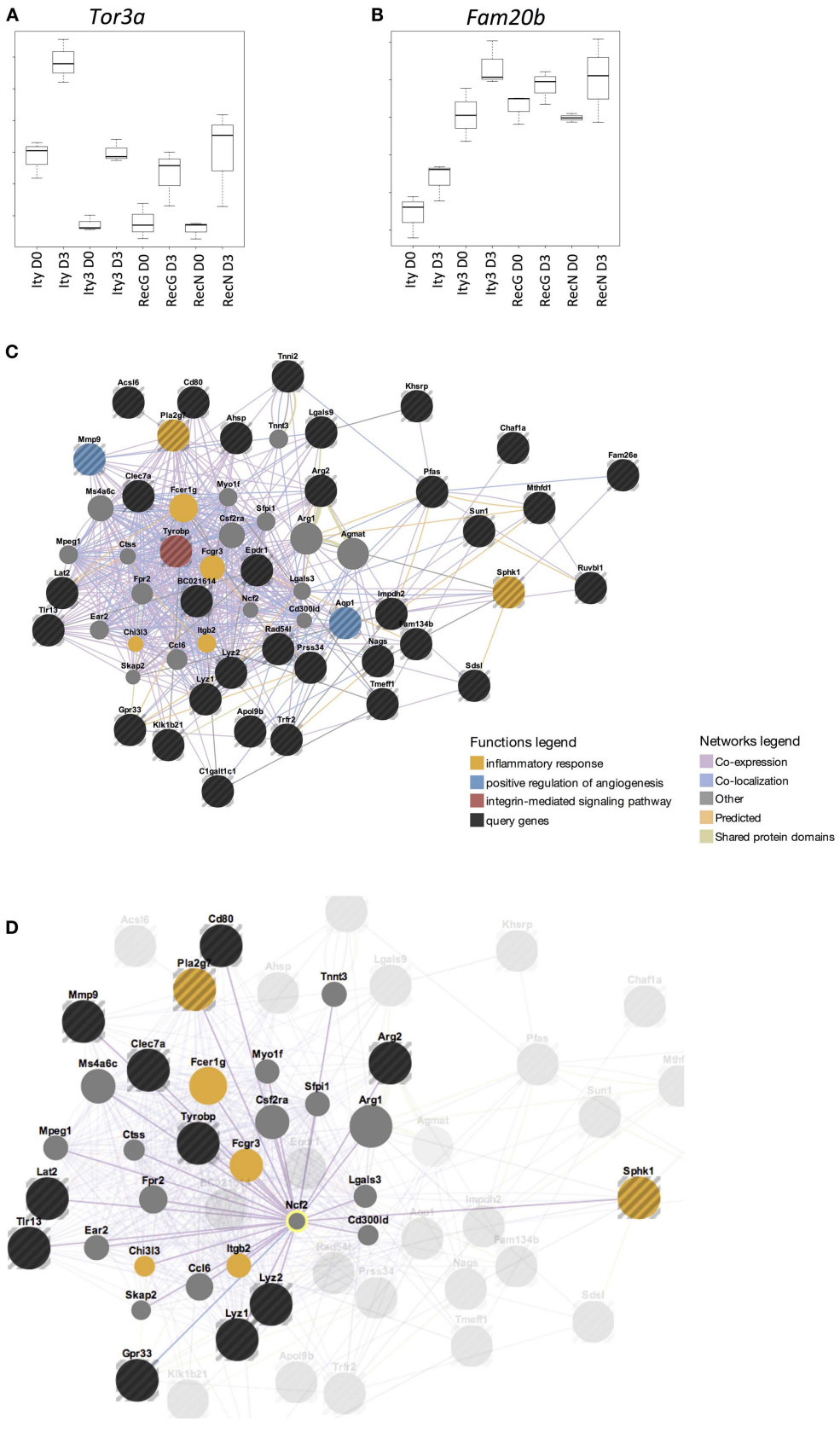

FIGURE 4 | Continued

FIGURE 4 | Continued
Genes that are differentially expressed in all susceptible and intermediate strains are under the influence of *Ity3.1* sub-locus. Box plots of the expression pattern of two genes **(A)** *Tor3a* (torsin family 3, member A) and **(B)** *Fam20b* (family with sequence similarity 20, member B) are shown as examples to illustrate the expression pattern seen in the gene list provided in Table S2I in Supplementary Material. This list of genes show a similar expression in *Ity3*, *Ity3.RecG*, and *Ity3.RecN* and highlights the complex nature of the *Ity3* locus as there are multiple genes in which expression is influenced by the combination of the two sub-loci. The genes that show a similar expression pattern in *Ity3*, *Ity3.RecG*, and *Ity3.RecN* were studied using GeneMania and the results are shown **(C,D)**, with the query genes being highlighted in black. Genes, which are known to be co-expressed, co-localized, have shared domains or predicted interactions with the list of genes in Table S2I in Supplementary Material are shown. The functional categories, which are enriched within this gene list, are inflammatory response, angiogenesis, and integrin mediated signaling pathways shown in yellow, blue, and red, respectively. Genes that were not differentially expressed but important in these pathways are shown in gray. **(D)** Co-expression of query genes, as well as other genes within these pathways, with *Ncf2* is shown.

functional categories (**Figure 4C**) such as inflammatory response and regulation of angiogenesis. A large proportion of the genes were either co-expressed, co-localized, or have shared domains or predicted interactions with *Ncf2* (**Figure 4D**). We have previously shown that the MOLF/Ei allele at the *Ity3.1* locus contributed the strongest effect on susceptibility to *Salmonella* infection and was responsible for high bacterial burden and low ROS and cytokine production (16). The fact that a number of genes differentially regulated in *Ity3*, *Ity3.RecN*, and *Ity3.RecG* strains, were co-expressed with *Ncf2*, supports the important contribution of the *Ity3.1* locus on the pathogenesis of infection in MOLF/Ei and its interaction with the other sub-locus *Ity3.2* to enhance the impact of *Ncf2* on ROS production.

Selp IS A CANDIDATE GENE FOR *Ity3.2*

We next studied genes showing a similar regulation pattern in *Ity3* and *Ity3.RecN* to understand the pathways differentially regulated in *Ity3.2* and identify potential candidate genes for the *Ity3.2* locus. There were only 14 genes that showed a similar expression pattern in *Ity3* and *RecN* (**Figure 5**). Of these 14 genes, 12 are located on chromosome 1 and 6 genes (*F5*, *Pbx1*, *Cacybp*, *Bc055342*, *Selp*, and *Vamp4*) lie within the genomic region harboring *Ity3.2* (**Table 1**). Sequence variations have been reported between the MOLF/Ei and C57BL/6J in coagulation factor *F5*, the cDNA *BC055324* and selectin P (*Selp*). The coagulation factor V is synthesized by the liver and is involved in the acceleration of prothrombin to thrombin conversion (32). Coagulation Factor V deficiency leads to a bleeding disorder associated with mild to severe hemorrhagic symptoms (33). The cDNA *BC055324* is poorly characterized and its function is not known. The *Selp* gene encodes for an adhesion molecule that mediates the recruitment of immune cells to the site of inflammation and is critical for the host immune response to infection making this gene an attractive candidate gene for *Ity3.2*.

We further evaluated the candidacy of *Selp* as the gene underlying *Ity3.2* using sequence analysis and complementation assay *in vivo*. *Selp* encodes for a protein of 768 amino acids with a C-type lectin domain, an EGF domain and 8 complement control protein (CCP) modules [or as short consensus repeats (SCRs) functional domains]. We re-sequenced the coding region of *Selp* in C57BL/6J and MOLF/Ei mice and identified eight SNPs (**Table 2**), all of which are within the less homologous CCP domains, involved in protein recognition processes (34). The amino acid proline at position 205 is well conserved across 12 mammalian species and P205S is only observed in the DBA sub-strains, which share MOLF/Ei ancestry for this region of the genome (mouse phylogeny viewer) (35). In order to validate that the sequence variation in the MOLF/Ei *Selp* gene has an impact on susceptibility to infection with *Salmonella typhimurium* and to evaluate if *Selp* is indeed the gene underlying *Ity3.2*, we used an allelic complementation assay (see breeding scheme in Figure S1 in Supplementary Material). *Ity* ($Selp^{B6/B6}$) and *Ity3* ($Selp^{MOLF/MOLF}$) mice were crossed to $Selp^{-/-}$ knock-out mice and susceptibility to infection was assessed by survival analysis in F1 progeny with $Selp^{MOLF/-}$ and $Selp^{B6/-}$ genotypes. $Selp^{MOLF/-}$ mice were significantly more susceptible to infection than $Selp^{B6/-}$ mice and *Ity* controls (**Figure 6A**). We observed a lack of complementation in $Selp^{MOLF/-}$ mice with a mean survival time equivalent to $Selp^{-/-}$ animals (MST of 8.1 ± 0.18 and 8.3 ± 0.38, respectively), adding further support for the candidacy of *Selp* as the gene underlying the *Ity3.2* locus (**Figure 6A**). Although the $Selp^{-/-}$ mice showed a similar susceptibility compared to the $Selp^{MOLF/-}$ mice in terms of survival, their tissue bacterial burden was significantly lower when compared to $Selp^{MOLF/-}$, *Ity3*, and *RecG* mice (**Figure 6B**) suggesting that the *Ity3*.2 locus does not contribute significantly to the bacterial burden and that the high bacterial burden observed in $Selp^{MOLF/-}$ mice is rather the effect of the *Ity3.1* locus.

DISCUSSION

The current study was specifically designed to understand the pathways that are influenced by the *Ity3* locus using sub-congenic strains that exhibit different degrees of susceptibility to *Salmonella* infection. The global gene expression profile in the spleen was studied early during infection prior to a significant bacterial difference in the target tissue. This approach allowed us to identify networks, which are of importance in the early phases of innate immunity yet not influenced by the extent of bacterial burden in the spleen. We reported a group of genes, the majority of which are regulated by type I and type II IFN. These genes, such as the *Gbp*, *Oas*, *Ifitm* family members, are differentially regulated in all strains of mice during infection, and define a core transcriptional signature common to several strains of mice infected with *Salmonella* (15).

Additionally, we characterized a number of genes not located within the *Ity3* region that were differentially expressed in the susceptible *Ity3*, *Ity3.RecG*, and *Ity3.RecN* strains as compared to the resistant *Ity* strain. We showed that these genes are also co-expressed with *Ncf2*, further supporting the hypothesis that there

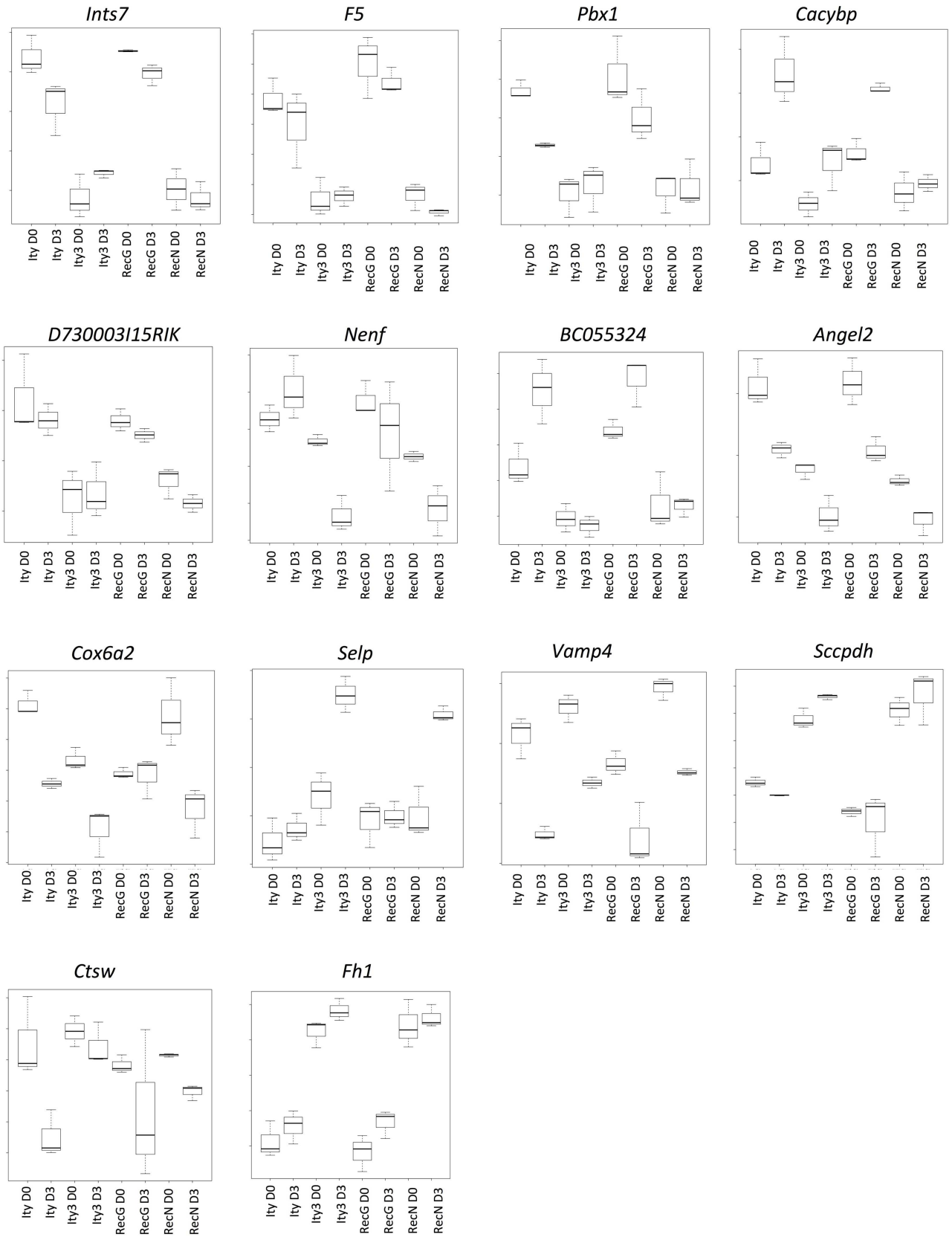

FIGURE 5 | Continued

FIGURE 5 | Continued
Genes under the influence of *Ity3.2*. List of genes showing a similar expression pattern in *Ity3* and *Ity3.RecN*, and different from *Ity* and *Ity3.RecG*. *Ints7* (integrator complex subunit 7), *F5* (coagulation factor V), *Pbx1* (pre B cell leukemia homeobox 1), *Cacybp* (calcyclin binding protein), *Nenf* (neuron derived neurotrophic factor), *Angel2* [angel homolog 2 (*Drosophila*)], *Cox6a2* (cytochrome c oxidase subunit VIa polypeptide 2), *Selp* (selectin, platelet), *Vamp4* (vesicle-associated membrane protein 4), *Sccpdh* [saccharopine dehydrogenase (putative)], *Ctsw* (cathepsin W), *Fh1* (fumarate hydratase 1).

Table 1 | List of known SNPs within differentially expressed genes in *Ity3* and *Ity3.RecN*.

Target	Variation	Chromosome	Location (bp)
DOWN-REGULATED			
Ints7	No exonic variation	1	191575734
F5	rs6271495	1	164151838
Pbx1	No exonic variation	1	168153527
Cacybp	No exonic variation	1	160202367
D730003i15rik	No exonic variation	1	191224474
Nenf	No exonic variation	1	191306789
Bc055324	rs30651611	1	163945993
Angel2	No exonic variation	1	190925112
Cox6a2	No exonic variation	7	128205436
UP-REGULATED			
Selp	rs30667849	1	164115264
Fh1	rs13465421	1	175600374
Vamp4	No exonic variation	1	162570515
Sccpdh	No exonic variation	1	179668210
Ctsw	No exonic variation	9	5465240

Table based on data from the Mouse Genome Informatics (MGI) website.

Table 2 | Exonic variation in the MOLF/Ei allele of the *Selp* gene.

	Base pair change	Amino acid change	Domain
Selp	730G > T	V202F	Sushi/CCP/SCR
	739A > G	N203D	Sushi/CCP/SCR
	745C > T	P205S	Sushi/CCP/SCR
	620C > T	H207Y	Sushi/CCP/SCR
	841G > A	G239S	Sushi/CCP/SCR
	1135G > A	V337I	Sushi/CCP/SCR
	1775A > C	N550T	Sushi/CCP/SCR
	1831A > G	I569V	Sushi/CCP/SCR

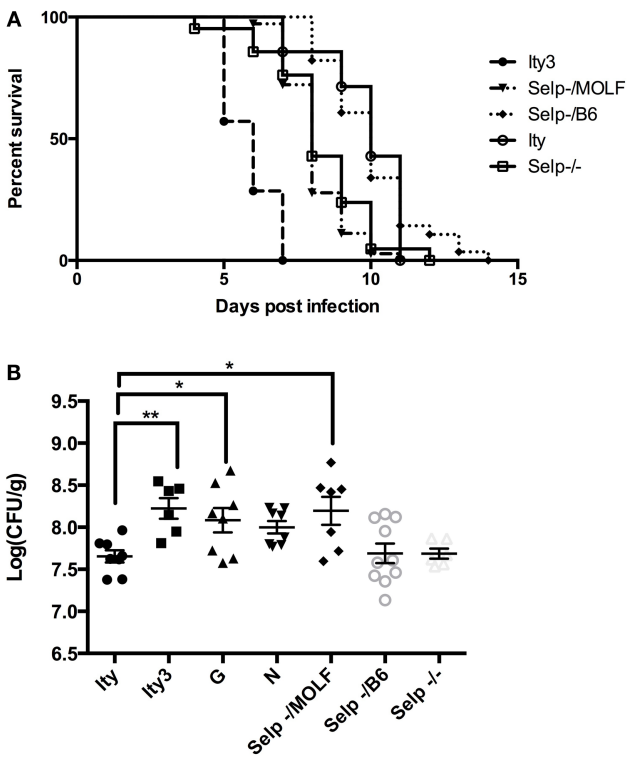

FIGURE 6 | Allelic complementation between *Ity3* congenic mouse and *Selp* deficient mice. In order to assess the impact of the MOLF/Ei *Selp* locus, $Selp^{-/-}$ mice were crossed with *Ity* and *Ity3* mice to generate mice carrying the knock-out allele complemented by the MOLF/Ei allele at *Selp* or C57BL/6J allele at the *Ity3* locus. **(A)** Survival curves of the congenic *Ity* ($n = 7$), *Ity3* ($n = 7$), knock-out $Selp^{-/-}$ ($n = 21$), and $Selp^{-/MOLF}$ ($n = 36$), $Selp^{-/B6}$ ($n = 56$) mice after infection with *Salmonella typhimurium*. The $Selp^{-/-}$ and $Selp^{-/MOLF}$ show a similar curve after infection with *Salmonella typhimurium*, both of which are more susceptible than the control *Ity* congenic mice and $Selp^{-/B6}$, but more resistant than the mice carrying the entire *Ity3* locus (*Ity3*). **(B)** Bacterial burden in the spleen of congenic *Ity* ($n = 8$) and *Ity3* ($n = 6$), sub-congenic *Ity3.RecG* ($n = 8$), and *Ity3.RecN* ($n = 8$) and $Selp^{-/-}$ ($n = 6$) and compound heterozygous ($n = 7$ for $Selp^{-/MOLF}$ and $n = 10$ for $Selp^{-/B6}$ mice after infection).

is an influence of the *Ity3.1* sub-locus on other segments of the genome. These results highlight the importance of the region of distal chromosome 1 carrying *Ity3*, a region enriched in QTLs. Over 80 QTLs are listed at the mouse genome database (36) and a number of cis and trans eQTLs have been characterized (37). Among them, several QTLs are involved with complex inflammatory reaction, such as graft vs. host disease (38) lupus (39), modifier of LPS-response (40), and susceptibility to tuberculosis (41).

We have reported previously that *Ity3* influences ROS production during infection (13) and this effect was mapped recently to a small sub-region named *Ity3.1*, which harbors the gene *Ncf2*, a subunit of the NADPH complex. (16). ROS produced by NADPH has been shown to affect a number of pathways, which are important in innate immunity including bacterial killing within the phagolysosome where ROS interact with other ions such as chloride to form toxic agents (HOCL) or can convert into hydroxyl radicals that are toxic for bacteria (42). ROS production has also been shown to influence immune cell recruitment, activation, and survival (43), and lead to translational activation of NF-κB (44). NADPH oxidase derived ROS is also a key regulator of autophagy and autophagy regulation during pathogen invasion (45). In addition, NADPH oxidase activation contributes to the recognition and removal of apoptotic neutrophils (efferocytosis) by macrophages (46–48). Therefore, an imbalance in NADPH

produced ROS can lead to increased inflammation, which can be deleterious to the host.

Recent studies have shown that TNF, as well as other cytokines through NF-κB signaling induces transient increase in ROS level in endothelial cells, which results in cell surface expression of *Icam* and *Selp* (49–52). This effect has further been studied *in vivo*, where mice lacking the $p47^{phox}$ (*Ncf1*) subunit of the NADPH complex have reduced expression of VCAM-1, ICAM-1, SELP, and SELE in the vascular cell walls (53, 54). In the current study, we identified *Selp* as a candidate gene for the locus *Ity3.2* and used an allelic complementation assay to provide genetic evidences that *Selp* is indeed a strong candidate for the *Ity3.2* locus. In MOLF/Ei mice susceptibility to infection as explained by the *Ity3* locus could be attributed to the individual effect of *Ity3.1* and *Ity3.2* sub-loci but also to the cooperation between these two sub-loci as explained by the potential impact of low activity of NADPH oxidase (16) on *Selp* function by reducing its expression (current paper).

We also illustrated that mice carrying a MOLF/Ei allele at the *Ity3.1* locus have higher expression of a number of genes playing a role in cell cycle, DNA binding, and cytoskeletal pathways. There is a growing body of evidence discussing the link between ROS, cell-cycle progress and arrest. As discussed by Martindale (55), ROS can have multiple effects on the cell cycle, depending on the amount and type of ROS. They suggest that low doses of ROS may cause proliferation while high doses of ROS can lead to apoptosis and cell death. In our model, it is possible that reduced levels of ROS in susceptible animals could lead to cell growth arrest, hence providing a more favorable niche for bacteria to replicate.

Another pathway influenced by ROS production, is up-regulated by *Ity3.1* and regroups genes involved in heme biosynthesis. Increased expression of genes within the heme biosynthesis pathway could result in increased free heme within the cells, which can act as a potent cytotoxic pro-oxidant (56). Free heme has also recently been shown to trigger necroptosis in macrophages (57). Therefore, it is possible that the increased expression of the heme biosynthesis pathway observed in susceptible mice is a mechanism that compensates for the low ROS levels.

In conclusion, our study highlights the complex nature of multi-loci interaction in the wild-derived MOLF/Ei response to *Salmonella* infection. We highlighted the role of low ROS and cytokine production in reduced survival of mice carrying the *Ity3.1* locus, and the importance of the *Ity3.2* locus, which synergistically led to increased susceptibly of the *Ity3* mice. We have also shown that several pathways identified in strains *Ity3*, *Ity3.RecN*, and *Ity3.RecG*, are influenced by *Ncf2*. Furthermore, the *Ity3.1* sub-locus has additional effects, which have not previously been characterized, in expression of genes involved in cell-cycle arrest and hematopoiesis. Lastly, we propose a hypothesis that the combined effects of low ROS production by the MOLF/Ei *Ity3.1* locus together with the impact of *Selp* MOLF/Ei allele at *Ity3.2* influences the host survival after infection with *Salmonella typhimurium*.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fimmu.2014.00375/abstract

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Pathobiology of *Salmonella*, intestinal microbiota, and the host innate immune response

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Salmonella is a relevant pathogen under a clinical and public health perspective. Therefore, there has been a significant scientific effort to learn about pathogenic determinants of this pathogen. The clinical relevance of the disease, associated with the molecular tools available to study *Salmonella* as well as suitable animal models for salmonellosis, have provided optimal conditions to drive the scientific community to generate a large expansion of our knowledge about the pathogenesis of *Salmonella*-induced enterocolitis that took place during the past two decades. This research effort has also generated a wealth of information on the host immune mechanisms that complements gaps in the fundamental research in this area. This review focus on how the interaction between *Salmonella*, the microbiota and intestinal innate immunity leads to disease manifestation. As a highly successful enteropathogen, *Salmonella* actively elicits a robust acute intestinal inflammatory response from the host, which could theoretically lead to the pathogen demise. However, *Salmonella* has evolved redundant molecular machineries that renders this pathogen highly adapted to the inflamed intestinal environment, in which *Salmonella* is capable of outcompete resident commensal organisms. The adaptation of *Salmonella* to the inflamed intestinal lumen associated with the massive inflammatory response that leads to diarrhea, generate perfect conditions for transmission of the pathogen. These conditions illustrate the complexity of the co-evolution and ecology of the pathogen, commensals, and the host.

Keywords: *Salmonella*, innate immunity, intestinal microbiota, inflammation, enteritis, symbiosis

INTRODUCTION

Salmonella infection or the disease associated with it, salmonellosis, is most often characterized by enteritis. However, host restricted serotypes tend to induce higher levels of bacteremia, while some human restricted serotypes cause a systemic disease with mild enteric symptoms. All infections in warm blooded animal species and humans are due to one single *Salmonella* species, namely *Salmonella enterica* subsp. *enterica*, which includes more than 2,400 serotypes (1). Currently, there is an effort to reclassify *S. enterica* according to genotypes (based on multilocus sequence typing – MSLT) rather that serotypes. MSLT may be more accurate for predicting pathogenicity and host preferences (2). Although human restricted serotypes (i.e., Typhi and Paratyphi) cause a systemic disease named typhoid fever, several other serotypes, so-called "non-typhoidal *Salmonella*" (NTS) are capable of infecting human patients causing primarily an enteric disease characterized by enteritis and diarrhea. Most of the studies on *Salmonella* enteropathogenesis have been performed with serotype Typhimurium, therefore, unless stated otherwise, this review refers to *Salmonella typhimurium*.

Our understanding of the pathogenic mechanisms of NTS has markedly advanced over the past 20 years. Two important steps were crucial for achieving such advancement: (i) genetic manipulation of the pathogen that allowed researchers to dissect several of the *Salmonella* virulence factors, and (ii) development and characterization of suitable experimental models. Thus, the most significant molecular mechanisms employed by *Salmonella* for invasion and intracellular survival in host cells have been deciphered. *Salmonella* actively invades intestinal epithelial cells. The invasion process requires several effector proteins that are translocated through the *Salmonella* pathogenicity island-1 (SPI-1)-encoded type III secretion system (TTSS) (3, 4). *Salmonella* is also capable of surviving intracellularly in phagocytic and non-phagocytic cells. Intracellular survival requires a second TTSS that is encoded by the *Salmonella* pathogenicity island 2 [SPI-2; (4, 5)].

In parallel to the progress in the field of molecular microbiology, experimental models, including epithelial, phagocytic, and other cell lines (6), as well as the development of animal models were instrumental for advancing in our knowledge on *Salmonella* enteropathogenesis (7, 8). Importantly, there are marked differences on how mammalian hosts respond to *Salmonella* (7). The mouse has been extensively used as a model for experimental infections. Importantly, marked differences in natural resistance has been demonstrated among mouse strains, which is associated with the resistant (e.g., strain 129sv) or susceptible (e.g., strains C57BL6/J and BALB/c) allele of the *Slc11a1* (formerly known as *Nramp1*) gene (9). However, inoculation of mice with *S. typhimurium* results in a systemic infection that is not associated with diarrhea (7), but resembles typhoid fever caused by *S. typhimurium* in human patients (10). Therefore, aside of a few experimental reports with non-human primates (11, 12), bovine experimental infections became very relevant in this context (13) since cattle respond to NTS infection by developing an enteric disease that is clinically similar to human NTS infections (13, 14).

Calves can be either orally infected (15) or subjected to surgical ligation of ileal loops that allow for a more precise assessment of early host responses (14). However, experimental studies performed in the 1980s have demonstrated that the absence of the intestinal microbiota has a profound impact on the outcome of infection in the mouse, rendering mice much more susceptible to infection (16). Furthermore, very early experimental studies have demonstrated that mice treated with streptomycin had an increased susceptibility to *Salmonella* (17), which allowed the development of a mouse model of *Salmonella*-induced typhlocolitis based on disruption of the intestinal microbiota by pre-treating the mice with streptomycin prior to challenge with *S. typhimurium* (18). This new model opened the opportunity to largely expand animal experimentation on *Salmonella*-induced intestinal inflammation, but it also clearly demonstrated the profound impact that the intestinal microbiota may have on the pattern of host response and outcome of infection.

The goal of this review is to discuss the advances in our knowledge on the innate intestinal immunity under the light shed by studies on the interaction between *Salmonella*, the intestinal microbiota, and the host.

INTERDEPENDENCE OF THE INTESTINAL MICROBIOTA AND THE IMMUNE SYSTEM

During the past few years, it has become increasingly clear that the intestinal microbiota plays a major role modulating intestinal mucosal immunity [reviewed by Ref. (19)]. Mammals coevolved with a complex population of commensal microorganisms that establish a mutually beneficial relationship to an extent that mammalians host more than 10^{14} microorganisms in the intestine (19). The significance of the microbiota for the development of the immune system is illustrated by the several immune defects that are observed in germ free mice, including decreased gut-associated lymphoid tissue, smaller mesenteric lymph nodes, and decreased antibody production, among other structural and functional deficiencies (19). It has been demonstrated that the host specific microbiota is required for full development of the mucosal immunity in the mouse (20). The Th-17 subset of T-cells is required for homeostasis and mucosal integrity, whereas the development of this cell population in the intestine requires the establishment of the microbiota, since germ free mice fail to develop Th-17 in the intestine (21). In a healthy individual, the microbiota prevents translocation of pathogenic microorganisms to the mesenteric lymph node thus preventing an undesirable immune response (22). Disruption of the microbiota (known as dysbiosis) due to antibiotic treatment favors translocation of even a non-invasive mutant *S. typhimurium* strain by phagocytes to the mesenteric lymph node (22).

In the past few years, a large number of relevant scientific reports have clearly established how the pathogen-associated molecular patterns (PAMPs) are recognized by their hosts (ranging from insects to mammalians) through pathogen recognition receptors (PRRs). However, a more recent wave of experimental evidences support the notion that molecules derived from the commensal microbiota are constantly sensed by host PRRs, which is a key step in establishing homeostasis [reviewed by Ref. (23)]. MyD88, a key adaptor protein for most TLRs (toll-like receptors), has been shown to play an important role in this context, since mice lacking MyD88 have a 100-fold increase in the number of bacteria associated with the intestinal mucosa (24). Therefore, considering that commensal microbiota is also sensed by PRRs, the term MAMPs, which stands for microbe-associated molecular patterns, has been proposed (25). Divergence between a PRR-mediated inflammatory response and PRR-mediated immune modulation and homeostasis is dependent on the concurrent presence of additional signals such as stimulation of cytosolic receptors by MAMPs (26). Importantly, in addition to sensing MAMPs, some of the cytosolic PRRs [i.e., Nod-like receptors (NLRs)] are capable of sensing signals associated with cell stress and damage, such as potassium influx, reactive oxygen species, membrane damage, etc. These signals are named danger-associated molecular patterns (DAMPs). Therefore, concomitant stimulation of extracellular PRRs and cytosolic PRRs by MAMPs or DAMPs allows the innate immune system to differentiate between stimuli from the commensal microbiota leading to homeostasis or pathogen triggered responses that lead to inflammation [reviewed by Ref. (27)].

While the establishment of the intestinal microbiota is a key event for immune maturation, conversely, immune cells in the intestine play an active role in shaping the composition of the microbiota, leading to homeostasis [reviewed by Ref. (28)]. For instance, the absence of $CD4^+$ T_{reg} cells results in an unregulated T-cell response against antigens from the microbiota, which causes intestinal inflammation (29). Mucosal antibodies, i.e., secretory IgA, also play a central role in shaping the microbiota. Impaired production of high affinity secretory IgA in the intestinal mucosa results in dysbiosis (30). Another very important component of this interaction between the host and microbiota are the intestinal epithelial cells (i.e., enterocytes, goblet cells, and Paneth cells). In addition to a physical barrier, structured by tight junctions between these cells that completely separate the apical from the basolateral compartment, the epithelium generates important factors that modulates expansion and composition of the microbiota. Goblet cells produce large amount of mucous that is a key element in homeostasis, while other cell types, particularly Paneth cells, generate antimicrobial peptides (31).

Interestingly, the influence of the microbiota is not restricted to the intestinal mucosal immunity, but it also impacts systemic immune sites. Antibiotic-induced dysbiosis results in impaired immune response against the influenza virus, while under these circumstances immunity is restored by rectal administration of PPR ligands, indicating that exposure of the intestinal mucosa to MAMPs is critical to modulating immunity (32). Indeed, there are experimental evidences of translocation of MAMPs from the intestine to systemic sites, where it modulates immune maturation, which indicates that the immune modulator role of the intestinal microbiota is not restricted to local tissues, influencing other distant immune organs (33). Interaction of the microbiota with the immune system is extremely complex, to the point that the microbiota may either favor or prevent the development of autoimmune disorders (34) as well as cancer development (35). Furthermore, the microbiota influences numerous other pleiotropic effects, both on pathologic events such as asthma, arthritis, inflammatory bowel diseases, obesity, and cardiovascular disease, as well as on physiological functions including organ morphogenesis,

intestinal vascularization, tissue regeneration, bone homeostasis, metabolism, and behavior (36).

SALMONELLA INTERACTION WITH THE INTESTINAL MICROBIOTA

As previously mentioned, earlier studies have clearly demonstrated that disruption of the intestinal microbiota by treating mice with streptomycin results in increased susceptibility to *Salmonella* infection (17). Furthermore, the intestinal microbiota has a protective effect against *Salmonella* infection in the mouse (16). These studies prompted Barthel et al. (18) to develop a very useful experimental model based on treatment of mice with streptomycin followed by challenge with *S. typhimurium*. This model has been extensively utilized by the entire field, since experimental infections were previously largely restricted to more expensive and labor intensive animal models such as oral infections in calves (15) or the bovine ligated ileal loops (14). Further studies demonstrated that *Salmonella* elicits an inflammatory response in streptomycin-treated mice that is pretty similar to that observed in *Salmonella*-infected germ free mice (37). While *S. typhimurium* infection in cattle triggers an acute inflammatory response that is characterized by massive infiltration of neutrophils (**Figure 1**) associated with variable degrees of necrosis, hemorrhage, erosion, and fibrinous pseudomembrane formation over the intestinal mucosa, particularly at the ileal Peyer's patches (14, 15), the same pathogen in the mouse does not elicit significant neutrophilic infiltration in the intestinal mucosa (7). Mice respond to *S. typhimurium* infection with a mild histiocytic infiltration, but in contrast they develop a marked systemic infection that is associated with lesions in the liver and spleen in the absence of diarrhea. Therefore, the development of the streptomycin-treated mouse model largely broadened the possibilities for *in vivo* experimental study of salmonellosis, allowing a marked worldwide expansion of animal experiments among several groups as well as genetic manipulation not just of the pathogen, but also of the host. Pretreatment with streptomycin results in a severe acute inflammatory response of the intestinal mucosa in response to *S. typhimurium* infection (**Figure 2**) (18). Although the original study that described this model demonstrated that streptomycin-treated mice have a much more efficient intestinal colonization with *S. typhimurium* (18), which suggests that the mechanism is likely due to lack of competition with components of the microbiota, this did not prove any direct cause or effect relationship between composition of the microbiota and the intrinsic nature of the innate intestinal immune response. Therefore, this model opened another extremely important area of investigation in this field, i.e., the role of the microbiota in the pathogenesis of NTS-induced enterocolitis.

Clinical treatment of human patients with antibiotics is recognized as a risk factor for subsequent *Salmonella* infection (38), which correlates well with what we have learned from the streptomycin-treated mouse model of *Salmonella* infection (18). However, the interaction of *Salmonella* with the microbiota is complex, and under certain circumstances pathogen and commensal may not necessarily have a mutually excluding relationship. For instance, a recent study demonstrated that carbohydrates metabolized by commensal microorganisms may serve as energy source for *Salmonella*. In that study, *Bacteroides thetaiotaomicron*, which encodes sialidase that is required to release sialic acid from glycoconjugates, but does not have the enzymatic machinery to utilize sialic acid as a carbon source, generates free sialic acid, whereas *S. typhimurium* that lacks sialidase is capable of catabolizing this carbohydrate (39). *S. typhimurium* can also metabolize fucose generated in a similar manner. Therefore, members of the commensal microbiota are capable of releasing carbon sources that themselves cannot utilize, but that can be used as energy source by *Salmonella* (39). This process is thought to play a role in post antibiotic expansion of enteropathogens (39).

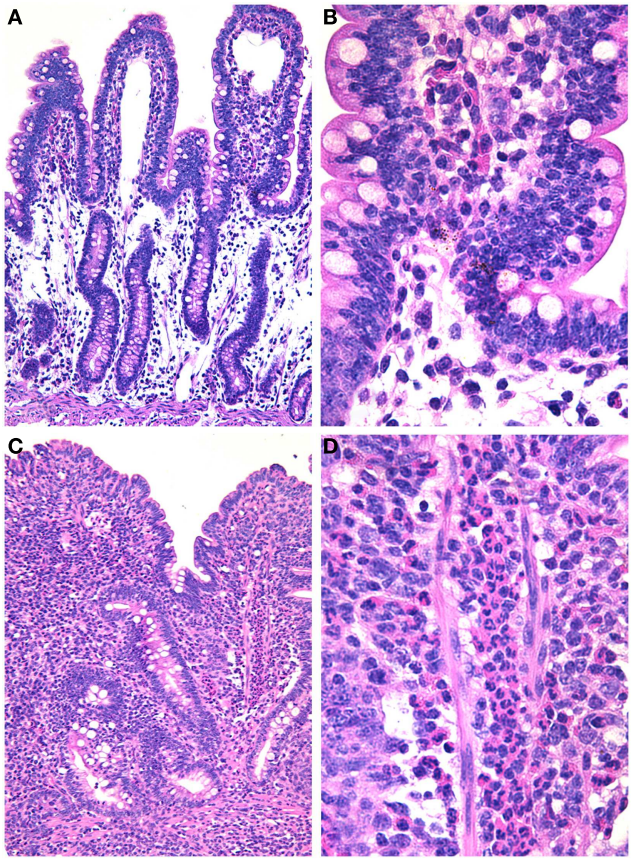

FIGURE 1 | *Salmonella*-induced enteritis in experimentally infected ligated ileal loops in calves. **(A)** Uninfected loop with no inflammatory reaction; 10× objective. **(B)** Higher magnification of uninfected loop; 40× objective. **(C)** *Salmonella*-infected loop with a severe and diffuse inflammatory infiltrate and blunting of the villi; 10× objective. **(D)** Higher magnification showing a diffuse and severe infiltration of neutrophils; 40× objective. Hematoxylin and eosin.

Susceptibility to different enteric pathogens is highly variable among different age groups. Interestingly, these differences in susceptibility may at least in part be related to changes in the composition of the intestinal microbiota [reviewed by Ref. (40)]. During early infancy the microbiota is highly dynamic, whereas in adults it is much more stable and composed predominantly by the phylum Bacteroidetes and Firmicutes (approximately 95% of the microbiota), and elderly tend to have a predisposition to mild inflammation in the intestinal mucosa and decrease in the relative

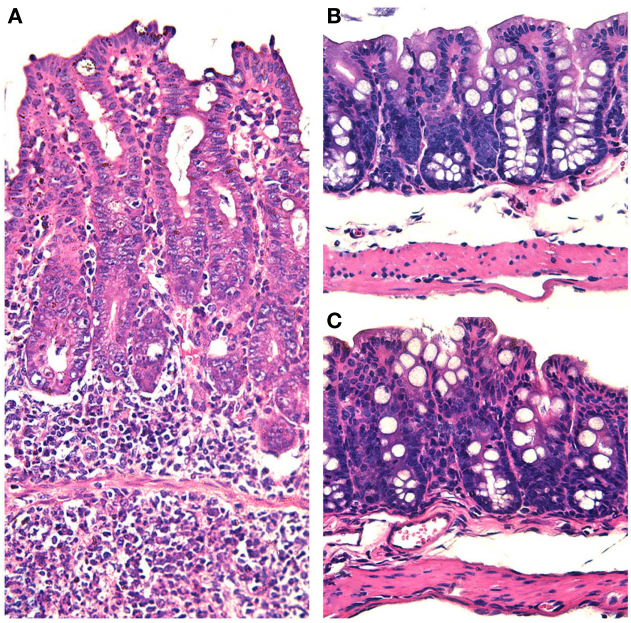

FIGURE 2 | *Salmonella*-induced typhlitis in mice with dysbiotic microbiota due to streptomycin treatment. **(A)** Marked thickening of the cecal wall with edema and increased cellularity due to a diffuse infiltration of inflammatory cells in a mouse with dysbiosis (pre-treated with streptomycin) and intragastrically infected with *Salmonella enterica* serotype Typhimurium. **(B)** Section of the cecum from a mouse intragastrically infected with *Salmonella enterica* serotype Typhimurium in the presence of a normal microbiota with no histopathological changes. **(C)** Section of the cecum from a healthy non-infected mouse. Note that all micrographs have the same magnification 20× objective. Hematoxylin and eosin.

abundance of Actinobacteria (essentially *Bifidobacteria*) and Firmicutes (40). Importantly, early and late stages of life, when the intestinal microbiota is less stable, with relatively lower numbers of Bacteroidetes and relatively higher numbers of gamma Proteobacteria, correspond to the period of higher susceptibility to some enteric pathogens (40).

With an elegant experimental approach, Chung et al. (20) demonstrated that germ free mice associated with human intestinal microbiota have increased susceptibility to *Salmonella* when compared to mice that had been associated with a normal mouse microbiota, indicating that under those experimental conditions the mouse microbiota is more protective against *Salmonella* than the human counterpart. This somewhat parallels the manifestation of *Salmonella*-induced intestinal pathology in these two host species, supporting the notion that the human microbiota may favor *Salmonella*-elicited intestinal inflammation, whereas the murine microbiota impairs the ability of *Salmonella* for triggering a host inflammatory reaction (7), which may be due to a lower antagonistic potential of the human microbiota when compared to that of mice.

An increasing number of experimental evidences points toward the notion that *Salmonella* has evolved multiple mechanisms by which it can overgrow members of the microbiota under conditions of an inflamed intestine (41). Several studies have identified *Salmonella* effectors, among other bacterial factors, that play a role in triggering host inflammation in the intestine (42). *Salmonella*-induced enteropathogenesis is strongly associated with the ability of the pathogen to invade epithelial cells and the intestinal mucosa. Therefore, five effector proteins translocated through the (SPI-1)-encoded TTSS, namely SipA, SopA, SopB, SopD, and SopE2, are required for invasion and enteropathogenesis (43). Earlier studies have demonstrated that *Salmonella* has a competitive advantage over the microbiota in the inflamed intestine, whereas such advantage does not take place in the absence of inflammation (44). Quite a few mechanisms by which *Salmonella* takes advantage of intestinal inflammation have emerged recently. Lipocalin-2, a host antimicrobial peptide, is generated in the inflamed intestine in response to IL-17 and IL-22, whose production is triggered by *Salmonella* infection. This peptide prevents iron acquisition by intestinal microorganisms. It binds enterobactin, a siderophore produced by several enteric bacteria. However, *Salmonella* produces salmochelin (in addition to enterobactin), another siderophore that is not bound by lipocalin-2. Thus, under conditions of inflammation and abundance of lipocalin-2, *Salmonella* has a competitive advantage over other intestinal bacteria (45). Iron deprivation in the inflamed intestine induces expression of colicin Ib by *Salmonella*, which is a bacteriocin active against other *Enterobacteriaceae*, providing additional competitive advantage to *Salmonella* against part of the commensal microbiota under inflammatory conditions (46). Among other mechanisms by which *Salmonella* overgrow the commensal microbiota in the inflamed intestinal environment is based on its ability to acquire microelements, including zinc (47). In the inflamed intestine, calprotectin produced by neutrophils inhibits bacterial growth by sequestering zinc. However, *Salmonella* is capable of evading this host protective mechanism by expressing a high affinity zinc transporter named ZnuABC (47).

Another striking example of *Salmonella* adaptation to intestinal inflammation was provided by Winter et al. (48), who demonstrated that the inflamed intestinal environment provides a respiratory electron acceptor for *Salmonella*. Tetrathionate has been used as an enrichment medium for *Salmonella* isolation *in vitro* from samples containing competitive microbes since the 1920s. Reactive oxygen species generated during the inflammatory process triggered by *Salmonella* itself, oxidizes endogenous thiosulfate to generate tetrathionate, which can then be utilized as an anaerobic respiratory electron acceptor by *Salmonella* (48). This mechanism provides competitive advantage for *Salmonella* in the inflamed intestine while members of the microbiota perish due to environmental changes resulting from the massive *Salmonella*-induced inflammatory response. *Salmonella*-induced inflammation is associated with detachment of large numbers of enterocytes from the mucosa (14), Interestingly, ethanolamine derived from phosphatidylethanolamine, the most abundant phospholipid in membranes of detached enterocytes, can be utilized by *Salmonella* under anaerobic conditions using tetrathionate as electron acceptor in the inflamed gut (49). In addition to tetrathionate respiration, the effector protein SopE induces nitrate production by the host, which favors growth of *Salmonella* by allowing anaerobic nitrate respiration (50). Neutrophil-derived elastase,

which is abundant in the inflamed intestine, suppresses components of the commensal microbiota, favoring intestinal growth of *Salmonella* (51). Mechanisms of *Salmonella* adaptation to the inflamed intestinal environment have been recently reviewed by Winter and Bäumler (52).

As a component of the innate host immune response, the inflammatory process should be seen as a host mechanism for preventing the spread of infection, which to some extent is completely correct, since in the absence of a neutrophilic response, *Salmonella* tends to spread more efficiently to systemic sites of infection, both in the mouse (53) as well as in cattle (54). These experimental observations parallel clinical disease since serotype Typhi that causes systemic infections does not elicit a significant intestinal neutrophilic response (10). However, as paradoxically as it may first seem, *Salmonella* evolved to take advantage of the host intestinal inflammatory response. Together, the studies discussed above clearly support the notion that *Salmonella*-induced inflammation is part of this pathogen strategy to create a highly favorable environment in the intestinal lumen for its own multiplication. However, *Salmonella* is a facultative intracellular pathogen, and that interaction with host cells is a determinant of the pathogenic capacity of this organism. Earlier studies strongly focused on the interaction of *Salmonella* with different host cell types, both *in vitro* and *in vivo* (6), missing a very important aspect of the big picture, which is the fact that only a fraction of the *Salmonella* population in a given host actually invades the mucosa during the acute phase of infection, while most of the organisms remain in the intestinal lumen (41). Excessive invasion of the intestinal mucosa by a larger fraction of the population of *Salmonella* could not be desirable under the pathogen point of view, since once within the host tissues, *Salmonella* is exposed to several efficient bactericidal mechanisms. This may explain the role of the SptP effector protein that reverses some of the molecular mechanisms used by *Salmonella* to invade intestinal epithelial cells (55).

Summarizing, *Salmonella* uses a *kamikaze* strategy based on a small fraction of its infecting population actively invading and triggering a massive acute inflammatory response. While this acute neutrophilic response may effectively restrict the infection mostly to enteric sites, largely preventing survival of invasive bacteria, and therefore preventing systemic dissemination of the pathogen, it also creates an intraluminal intestinal environment that favors the remaining larger fraction of the pathogen population that stays in the intestinal lumen, being able to multiply and effectively transmit the infection to the next host.

MANIPULATION OF THE MICROBIOTA FOR PROPHYLACTIC AND THERAPEUTIC PURPOSES

A thorough review of prophylactic and therapeutic approaches to modulate the function and/or composition of the microbiota is completely beyond the scope of this article. However, under a clinical point of view, it is relevant to point out some of the advances in this area. Clinical applications of probiotic and prebiotic has been recently reviewed by Vieira et al. (56). Probiotics are defined as live microorganisms which when administered in adequate amounts confer health benefits to the host [FAO/WHO, 2002 FAO/WHO Working Group, Guidelines for the Evaluation of Probiotics in Food (2002). London, ON, Canada]. The notion of probiotic has been developed long time ago with the original observations of Metchnikoff in the beginning of the twentieth century, who identified microorganisms, particularly *Bacillus bulgaricus* (currently named *Lactobacillus bulgaricus*), which has beneficial effects on health and was the foundation of the yogurt industry (57). Probiotics, including different formulations and several different microorganisms in variable combinations, such as *Saccharomyces boulardii*, *Bifidobacterium* spp., *Streptococcus thermophilus*, *Lactobacillus* spp., *Escherichia coli* strain Nissle 1917, among several other microorganisms have been extensively used experimentally or therapeutically for treating enteric diseases with predominantly positive outcomes (56). However, particularly in immune compromised patients, the risk of sepsis should be taken in account when electing a probiotic therapeutic protocol (58). Prebiotics are food ingredients that are not digestible by the host and have favorable effects on specific components of the microbiota and intestinal homeostasis, although this concept may be expanded to include other food ingredients that do not completely fit the criteria for a prebiotic, but have similar effects, such as dietary fibers. Therapeutic or prophylactic combinations of probiotics and prebiotics are termed symbiotics (56).

A similar concept is linked to the ancient therapeutic practice of adoptive transfer of commensal microbiota from healthy individuals to patients with enteric diseases, particularly those associated with antibiotic therapy, which may be successful under certain conditions (59).

Specifically considering salmonellosis, there are experimental evidences indicating that probiotics may have a protective effect in mice experimentally challenged with *Salmonella*. Both germ free and conventional mice pre-treated with *Saccharomyces cerevisiae* UFMG 905 had lower levels of *S. typhimurium* dissemination upon experimental infection (60). Similarly, *Lactobacillus acidophilus* has protective effects against *S. enteritidis* infection in the mouse (61). Although it is not clear whether probiotics will ever have useful therapeutic applications in human patients infected with *Salmonella*, these experimental studies are relevant since probiotics and prebiotics have a significant potential for the animal industry, particularly for poultry and pigs. In food producing animal species, probiotics and prebiotics may prevent a high burden of *Salmonella*, thus mitigating the risk of transmission, with the additional significant benefit of decreasing the need and therefore the exposure of food producing animals to antibiotic treatment and growth promoters, which prevent emergence of antibiotic-resistant strains of pathogens. Indeed, several probiotics as well as food additives have been extensively studied under field conditions, but the results are highly variable, and strongly influenced by management, nutrition, environmental conditions, and obviously the levels of *Salmonella* challenge. Therefore, a general recommendation or a well-established protocol for probiotic or prebiotic prevention of *Salmonella* infection in farm animals is still unavailable (62, 63).

CONCLUDING REMARKS AND PERSPECTIVES

Since the first identification of microorganisms of the genus *Salmonella* in the beginning of the last century, a large body of knowledge has been accumulated regarding microbiological features of

the organism, disease manifestation in different host species as well as its epidemiological implications. However, it was only during the last decade of the past century that molecular tools became available for dissecting pathogenic mechanisms of *Salmonella*. These molecular approaches preceded more sophisticated animal models, and therefore the pioneer investigations on *Salmonella* pathogenesis pictured a pathogen highly specialized in invasion and induction of a host response, as if the pathogen was indifferent to the myriad of commensal microorganisms in the intestinal environment. A subsequent wave of well-designed studies began to reveal, at a mechanistic level, some of the interactions between *Salmonella* and the microbiota in the intestine. Currently, it is clear that the complexity of these processes is unimaginable at this point so this is still a broadly open field for scientific investigation. A deeper knowledge of the pathobiology of *Salmonella* in the context of the intestinal environment may certainly open new perspectives for therapeutic approaches as well as for controlling animal and human salmonellosis.

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The subtleties and contrasts of the LeuO regulator in *Salmonella* Typhi: implications in the immune response

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Salmonella are facultative intracellular pathogens. *Salmonella* infection occurs mainly by expression of two *Salmonella* pathogenicity Islands (SPI-1 and SPI-2). SPI-1 encodes transcriptional factors that participate in the expression of virulence factors encoded in the island. However, there are transcriptional factors encoded outside the island that also participate in the expression of SPI-1-encoded genes. Upon infection, bacteria are capable of avoiding the host immune response with several strategies that involve several virulence factors under the control of transcriptional regulators. Interestingly, LeuO a transcriptional global regulator which is encoded outside of any SPI, is proposed to be part of a complex regulatory network that involves expression of several genes that help bacteria to survive stress conditions and, also, induces the expression of porins that have been shown to be immunogens and can thus be considered as antigenic candidates for acellular vaccines. Hence, the understanding of the LeuO regulon implies a role of bacterial genetic regulation in determining the host immune response.

Keywords: LeuO, Typhi, OmpS1, OmpS2, H-NS, porins

INTRODUCTION

Salmonella enterica are Gram-negative bacterial pathogens capable of infecting human beings and other vertebrates, and causing substantial morbidity and mortality (1, 2). In human beings, most of *Salmonella* serovars can cause infections in the small intestine and hence gastroenteritis; yet a small percentage of *Salmonella* serovars can cause a systemic infection, such as typhoid fever by the Typhi serovar (3). Control of *Salmonella* infection is difficult, in part due to the capacity of the bacterium to tolerate environmental stress, to its widespread distribution, multiple drug resistance, and adaptability (4). They infect human beings and other animals by the fecal–oral route, via contaminated food and water.

After oral acquisition, *Salmonella* resists low pH in the stomach and colonizes the intestinal tract and some cells can disseminate to cause systemic infection of organs such as liver and spleen (1). *Salmonella* virulence factors as well as host immune responses are determinant in the infectious process developed in the pathology (5). *S. enterica* Typhimurium and Typhi serovars interact with host cells through the activities mainly of two type three secretion systems (TTSS), encoded in two pathogenicity islands, 1 and 2 (SPI-1 and SPI-2) (6, 7). While SPI-1 participates in bacterial cell entry into non-phagocytic epithelial cells, SPI-2 is required for intracellular maintenance of the bacteria in a specialized membranous compartment (8). *Salmonella* internalization is mediated by effectors encoded in SPI-1: SopE, SopE2, and SopB, which activate the Rho family of GTPases Rac1, Cdc42 and RhoG (9, 10). These bacterial effectors promote a transcriptional reprograming in host cells, which in turn leads to the expression of pro-inflammatory cytokines, which could be essential for the initiation of diarrhea, a hallmark of acute *Salmonella* infection. Recently, it has been observed that the expression of the pro-inflammatory cytokine interleukin 22 (IL-22) can be exploited by pathogens, such as *Salmonella*, to suppress the growth of their closest competitors thereby enhancing pathogen colonization of mucosal surfaces (11–13).

Upon infection of intestinal epithelial cells, early transcriptional host responses occur characteristically after the stimulation of the innate immune receptors (14). However, the *Salmonella*-induced responses are unique in that this pathogen is capable of stimulating them independently of innate immune receptors (12), which are largely inactive in the intestinal epithelial cells due to robust negative regulatory mechanisms (15–17). After internalization in epithelial cells, bacteria traverse the intestinal epithelium and can invade M-cells overlying Peyer's patches, as well as being captured by dendritic cells directly from the intestinal lumen (18).

Systemic infection requires intracellular survival and replication, while *Salmonella*-macrophage interactions are essential for bacterial virulence, disease, pathology and chronic infection (19–21). Immunity to intra-macrophage pathogens (i.e., *Salmonella*) requires the infected host to generate a robust and sustained CD4 Th1 response (22). *Salmonella* infection of inbred mouse strains induces a robust $CD4^+$ T-cell response that is essential toward protective immunity to secondary infection (23–27). *Salmonella* also induces $CD8^+$ T-cells and antibody responses that can contribute to the resolution of infection (25, 27, 28). The first study to successfully characterize *Salmonella*-specific $CD4^+$ T-cell clones identified the target antigen of these T-cells as an I-Ak epitope within the central hypervariable portion of bacterial flagellin encoded by the FliC gene (29). Subsequently, additional MHC class II epitopes were identified in the same protein and thus flagellin remains the most thoroughly defined target antigen in the *Salmonella* infection model (30, 31). Additional studies have

shown that immunization with flagellin provides a modest degree of protective immunity to *Salmonella* infection, usually defined by slightly lower bacterial counts or a delay in time to death after infection. Thus, flagellin is a well-defined target antigen of $CD4^+$ T-cells during *Salmonella* infection and this response contributes modestly to protective immunity *in vivo* (32, 33). Among other antigens, the outer membrane proteins (OMPs) are particularly important. In a murine model, the highly abundant OmpC and OmpF porins (34) can induce long-term antibody responses with high bactericidal capacity, and they even confer protection against challenge with *Salmonella* Typhi (35, 36).

THE LeuO GLOBAL REGULATOR IS AN LTTR

LeuO is part of the LysR-type transcriptional regulators (LTTRs), the largest family of transcriptional regulators in prokaryotes. In consequence, they regulate a wide variety of genes that are involved in a diversity of cellular functions such as biosynthesis of amino acids, catabolism of aromatic compounds, antibiotic resistance, oxidative stress response, nitrogen fixation, quorum sensing and virulence (**Figure 1**) (37–40). Many structural studies have shown an organization of an N-terminal DNA-binding domain (DBD) with a winged Helix-Turn-Helix (wHTH) motif; and a long linker helix (LH) involved in dimerization that connects the DBD with the C-terminal effector binding domain (EBD) or regulatory domain (RD) (37, 41–43). These regulators are proteins between 300 and 350 residues, mostly acting as transcriptional activators that bind to A–T rich DNA sequences in similar positions.

In the classical model of action, LTTRs activate the transcription of a divergent gene and repress their own transcription, independently of the presence of a co-inducer or effector (small signal molecule); although there are exceptions where no co-inducer is required and in most of these cases they act as repressors (37). Therefore, the members of the family have been described as dual regulators (44). Nevertheless, there are examples where the LTTR positively autoregulates its expression; and some LTTRs can have more gene targets that they activate or repress, involved in different cellular process, different from those divergently located with respect to the gene for the regulator (39). Even more, as addressed below, LeuO is an interesting case due to the fact that it can act as derepressor, and has been shown to have complex DNA-binding sites (45, 46).

LeuO HISTORY

The first report of the LeuO regulator was by the localization of the *leuO* gene between the *leuABCD* and *ilvIH* operons; upon which it was included in the LysR family due to its amino acid sequence

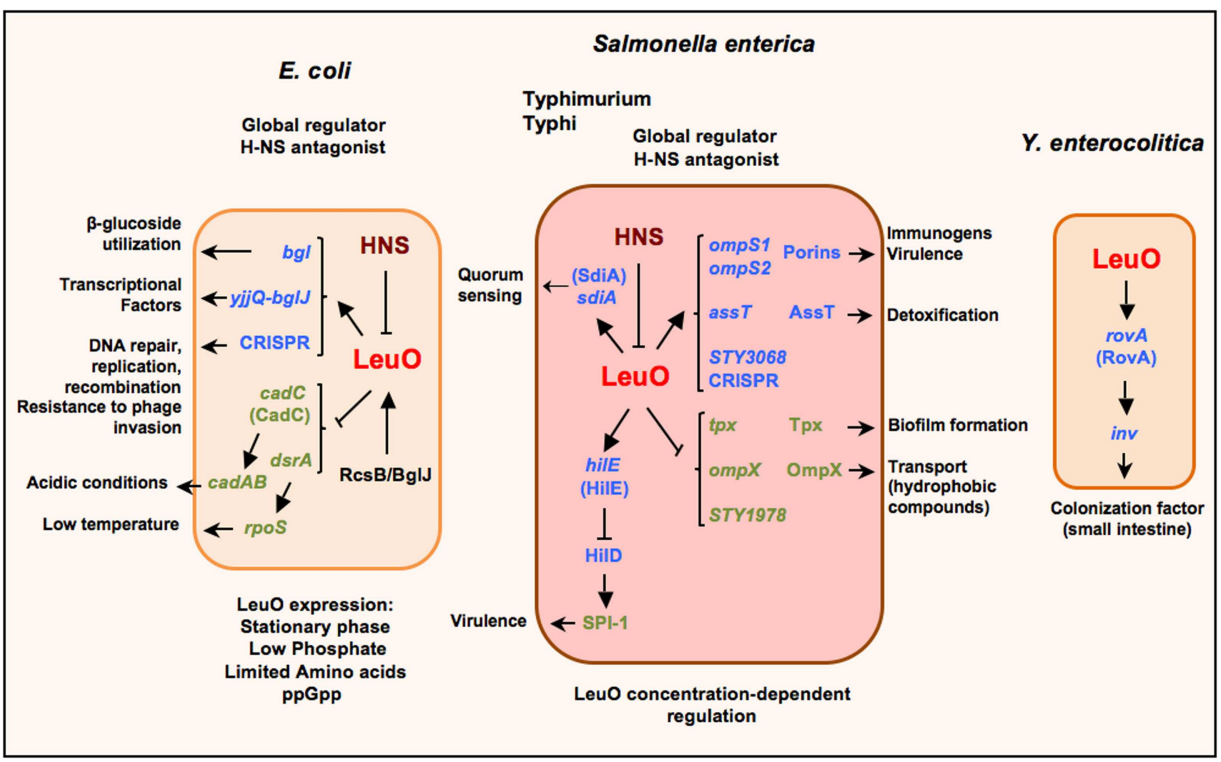

FIGURE 1 | Schematic representation of the LeuO regulon in *Escherichia coli, S. enterica* serovars Typhimurium and Typhi, and *Yersinia enterocolitica*. LeuO is a dual regulator that can induce the expression of several genes (arrows) and also is capable of repressing gene expression (lines). When acting as a repressor it has been suggested to function as a backup for H-NS; nevertheless in several cases LeuO acts as a derepressor of gene expression by displacement or prevention of H-NS repression. Recently, LeuO has been denominated as a global antagonist of H-NS in *E. coli* and in *S. enterica* serovar Typhimurium. The expression of *leuO* is repressed by H-NS, although there are some stress conditions when LeuO can be detected in *E. coli*. Also, in *Salmonella* it has been described as an interesting case of differential control of transcriptional regulation, which depends on LeuO concentration. Parentheses depict the proteins coded by the indicated genes. Small arrows denote the several functions for the LeuO-regulated genes.

similitude with other members of the family (47, 48). Based on the localization of its gene, LeuO was presumed to be a *leuABCD* regulator, although Leu auxotrophy was not observed in a *leuO* mutant strain (49).

Nevertheless, since the first report of LeuO as a transcriptional regulator, it was shown to be involved in the regulation of genes important for bacterial survival in stringent conditions (**Figure 1**). Thereby, when LeuO was overexpressed in *E. coli* it was found to repress *cadC*: this was the result of searching for genes that can complement an H-NS mutant strain, thus providing an insight about a relationship between LeuO and H-NS (50). CadC activates the *cadAB* operon, an important system expressed under acidic conditions (51). H-NS is a global regulator that acts as a nucleoid protein (52, 53). Later, LeuO was determined to reduce *rpoS* translation (which encodes S sigma factor) by repression of the small regulatory DsrA-RNA, who positively regulates *rpoS* translation, mainly at low temperature (54). Both *cadC* and *dsrA* are repressed by H-NS (55, 56). Interestingly, in both cases, LeuO indirectly represses the *cadAB* operon expression and RpoS translation.

According with a LeuO-dual role regulator, it was found to be a positive regulator of *bgl* and *yjjQ-bglJ* operons in *E. coli*. Later, it was demonstrated that LeuO counteracts H-NS repression (49, 57, 58). The *bgl* operon is involved in the utilization of some β-glucosides as salicin and arbutirin; and the *yjjQ-bglJ* genes encode for a transcriptional factor belonging to the LuxR family. These operons are repressed by H-NS in a wild type genotype (59) (**Figure 1**).

In several studies in *Salmonella* Typhimurium, a model called cis-acting promoter relay mechanism has been described that involves LeuO and DNA local supercoiling in a complex regulatory interplay, in a strain with a mutated promoter of *leuABCD* (p*leuO*-500), and a suppressor mutation in *topA* (60–62). In this complex regulatory mechanism, the Leucine-responsive regulator protein (Lrp) elicits changes in local DNA supercoiling by *ilvIH* promoter activation, exposing the *leuO* regulatory region upon which *leuO* can be transcribed (63–65). Also, there are H-NS binding sites in the regulatory region of *leuO*: hence the system appears to be repressed by changes in local supercoiling and LeuO prevents a cis-spreading of H-NS enhancing positive autoregulation and permits *leuABCD* transcription (66–69).

THE LeuO REGULATOR IN OTHER GRAM-NEGATIVE BACTERIA

Studies in *S. enterica* serovar Typhi (**Figure 1**) have shown that overexpression of LeuO induces the expression of two quiescent genes that encode for the OmpS1 and OmpS2 porins (70, 71). An interesting observation was that the LeuO concentration differentially affects *ompS1* and *ompS2* expression. The *ompS2* gene is expressed at lower concentrations of LeuO, whereas *ompS1* is expressed at higher concentrations where *ompS2* expression is repressed. Moreover, for the first time, in a detailed study of *ompS1* expression, LeuO was shown to exert an antagonist role toward H-NS (71). The relevance of this observation is that such function had not been reported for other LTTRs members until now. Interestingly, members of other transcriptional regulators families such as VirF (AraC/XilS), RovA (SlyA/Hor), and Ler (H-NS/StpA) have been described as antagonists of H-NS mainly on genes involved in virulence (72–74).

In a subsequent study to pursue more targets in *Salmonella* Typhi, LeuO was found to also positively regulate *assT* and STY3070; and negatively *ompX*, *tpx* and STY1978 (**Figure 1**). These genes are involved in a variety of cellular functions (75). AssT is a putative arylsulfate sulfotransferase that has been proposed to be involved in detoxification by transforming toxic phenolic derivatives into non-toxic compounds (76). The global regulators H-NS and LeuO regulate the *assT-dsbL-dsbI* cluster expression negatively and positively, respectively, and this regulation depends on specific growth conditions (77). STY3070 in *Salmonella* was later determined to be the *casC* gene of the CRISPR/Cas system; and its repression was found to depend also on Lrp, and its expression induced in minimal media independent of LeuO (78).

The CRISPR/Cas system in *Escherichia coli* has been involved in DNA repair, replication and recombination and is proposed to confer resistance to phage invasion in bacteria and archaea, thus the suggestion that it is an ancient defense mechanism (79). Interestingly, LeuO was shown to be an antagonist of H-NS in the CRISPR-system in *E. coli* (80). OmpX is an OMP that is homolog to PagC and Rck and Ail proteins of *Salmonella* and *Yersinia*, respectively. When overexpressed, it has been observed to increase sigma E activity; and the lack of *ompX* increased the tolerance to sodium dodecyl sulfate and antibiotics, thus appearing to affect the transport of hydrophobic compounds across the membrane (81–84). Tpx is a thiol peroxidase that codes for a periplasmic antioxidant enzyme that is induced during the exponential growth phase and during biofilm formation (85). It is important to notice that LeuO down-regulates proteins that are involved in the resistance to different pH conditions (83). Another down-regulated gene was STY1978, which codes for a hypothetical protein without an association to any cellular process until now. In this report, LeuO was denominated as a global regulator and opened the possibility that LeuO could have more targets depending on the growth conditions (75).

In *Y. enterocolitica*, LeuO was found to positively regulate *rovA* and, in turn, H-NS also negatively regulates its expression (86) (**Figure 1**). RovA is a MarA/SlyA type regulator that regulates *inv* gene expression in response to temperature and growth phase (87).

In *E. coli*, by SELEX screening, LeuO was found to regulate genes involved in sulfa drug sensitivity and to increase its own expression during transition into stationary phase and after a week of culture, where H-NS concentration decreased (**Figure 1**). Even more, a global antagonistic interplay between H-NS and LeuO was proposed, acting on some genes involved in stress response, such as cryptic chaperone/usher-type fimbriae. In addition, mutants in *leuO* and in some fimbrial genes were defective or altered in biofilm formation (88, 89).

In *S. enterica* serovar Typhimurium, LeuO was reported to increase *sdiA* expression in low levels (90) (**Figure 1**). SdiA is proposed to respond to signals produced by other organisms (91, 92) and recently was found to be active in gut in response to AHLs (*N*-acyl homoserine lactones) a quorum sensing signal produced by other species (93–96).

In a genomic study in *S. enterica* serovar Typhimurium, using ChIP-chip, the LeuO regulon members were extended to include SPI-1 (**Figure 1**) and SPI-2 genes. In addition, the differential binding of LeuO and regulation of genes was observed depending on the concentration of LeuO. Another important observation was the intragenic binding; hence opening the possibility that LeuO could act as a negative regulator preventing the progress of transcription or as nucleoid structure protein. The finding of LeuO co-binding at various sites with H-NS and RNA polymerase confirms the notion of the antagonist role of LeuO, although they could likely be acting together to regulate a large number of genes. Moreover, the possible interaction with RNA polymerase and H-NS would suggest another mechanism of LeuO regulation (45, 46).

In this respect, the structural properties of LeuO as an LTTR member have been initially explored: finding that it is active as a tetramer, that the mechanisms for induction and repression of gene expression appear to be different, and that there are relevant interactions between the N- and C-termini (97).

LeuO EXPRESSION CONDITIONS

In the *Salmonella* Typhi and *E. coli* wild type genomic backgrounds, LeuO expression is silenced by H-NS (unpublished data). Nevertheless, in *E. coli* and *Salmonella* Typhimurium, *leuO* expression has been detected when grown under stress conditions, especially in the stationary phase under nutrient limitation. Nevertheless, *leuO* is not under the control of *rpoS*; although its expression requires the presence of ppGpp in stationary phase (54, 63, 98, 99). Interestingly, LeuO was shown to be essential to restore cellular growth, after a 2-h delay in a media lacking isoleucine, valine, and leucine (100).

Also, LeuO expression was detected in a phosphate-restricted media (98); and recently it was shown that the expression of the *leuO* gene can be activated by the RcsB and BglJ regulators (58, 101)

LeuO HAS SEVERAL FUNCTIONS *IN VIVO*

Even though LeuO is expressed at very low level in standard laboratory conditions, it seems that *in vivo* it has a role in bacterial survival. In this manner, in a mouse and in a *Caenorhabditis elegans* model of infection, a *S. enterica* serovar Typhimurium *leuO* mutant showed to be attenuated in virulence. Also, in *Vibrio cholera*, biofilm formation was reduced in a deleted *leuO* strain (102–104).

Virulence attenuation in a murine model was reported for the *ompC ompF* double mutant (105). In addition, it has been observed that the OmpC and OmpF porins induced long-term antibody response with bactericidal capacity and conferred protection against challenge with *Salmonella* Typhi (35, 36). Nevertheless, these major porins are expressed at very high levels in standard laboratory conditions. In addition, strains lacking *ompS1* and *ompS2* are attenuated for virulence, suggesting that besides lacking the LeuO regulator the absence of OmpS1 and OmpS2 porins affected bacterial survival (103). Virulence attenuation of mutated strains in *leuO* and *ompS1* and *ompS2* quiescent genes offers evidence that they are expressed *in vivo*. Even though the specific role of these porins in *Salmonella* virulence is not clear, it has been shown that the major porins are passive diffusion channels of solutes, nutrients and toxins through the outer bacterial membrane that might allow bacteria to grow in different environments and to be resistant to drugs (106).

Recently it was found that OmpS1 and OmpS2 induced a strong immune response in the mouse, and a single dose conferred a significant protection against *Salmonella* Typhi. The immunostimulatory properties of OmpS1 and OmpS2 porins further reinforce the notion that they could be expressed following host infection. These studies are relevant because they open the possibility of using these porins as antigens for the development of vaccines against typhoid fever and other non-typhoidal salmonellosis (107).

Moreover, in a recent report it was shown that the activation of *leuO* transcription in *S. enterica* serovar Typhimurium represses expression of pathogenicity island 1 (SPI-1) and inhibits invasion of epithelial cells (108). Two different modes of action were found: the major one that involves the induction of *hilE* transcription by LeuO (**Figure 1**) and another one that was HilE-independent. HilE is a regulator encoded outside SPI-1 that represses *hilD* expression. HilD is one of the transcriptional factors encoded in SPI-1 that positively controls the expression of other genes in the island (109, 110). It has been suggested that LeuO repression of SPI-1 genes may occur under growth conditions where H-NS, for unknown reasons, has failed to perform such repression.

The possibility of LeuO acting as a backup for H-NS has two implications: one is that it could allow *Salmonella* to confront the hostile free-living conditions where SPI-1 gene expression has a high cost in bacterial growth; and two, it might ensure the specific, sequential, and appropriate level of SPI-1 gene expression in the intestine (111, 112). Due to the fact that H-NS in *Salmonella* is considered as a genome sentinel that silences horizontally acquired genes (113–115), LeuO could be acting as a backup regulator for H-NS, highlighting the subtleties and contrasts of the LeuO mode of action. Thus, the proposed role of LeuO as an activator or as a repressor depending on its concentration could explain this differential gene regulation.

LeuO is an example of a global regulator whose level of expression is an important issue, since this has an effect on its many regulated genes that are involved in a variety of cellular functions, such as virulence and bacterial survival. The levels of expression could thus have spatial and temporal consequences as well. In addition, knowledge of LeuO-regulated genes has been important in the study of the immune response induced by *Salmonella*, such as that elicited by the quiescent porins, which are protein components of the outer membrane. This has opened the possibility for the development of typhoid fever vaccines and perhaps as adjuvants for others vaccines.

It is intriguing that conditions known at present for LeuO expression are extreme and that in many studies it has to be overexpressed to analyze its function. Furthermore, no co-inducer of LeuO is known until now. These are some of the subjects that pose challenges for the future.

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Ubiquitination as an efficient molecular strategy employed in *Salmonella* infection

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The ubiquitin modification has various functions in the host innate immune system in response to the bacterial infection. To counteract the host immunity, *Salmonella* can specifically target ubiquitin pathways by its effector proteins. In this review, we describe the multiple facets of ubiquitin function during infection with *Salmonella enterica* Typhimurium and hypothesize how these studies on the host–pathogen interactions can help to understand the general function of the ubiquitination pathway in the host cell.

Keywords: ubiquitin, deubiquitinases, E3 ligases, autophagy, *Salmonella enterica* Typhimurium, *Salmonella*-containing vacuole, innate immune response, type III secretion system

INTRODUCTION

Salmonella enterica is an intracellular facultative anaerobe that is one of the leading causes of enteric diseases in the United States. Over 2500 serovars belonging to six sub-species of *S. enterica* have been identified. When ingested through contaminated food or water, *Salmonellae* cause disease syndromes such as typhoid, gastroenteritis, bacteremia, and chronic asymptomatic carriage (1, 2). *S. enterica* serovar Typhimurium, the causative agent of gastroenteritis has successfully evolved to cope with host defense mechanisms [reviewed in Ref. (2)]. The roles of different ubiquitin pathways in host innate immune system during *Salmonella* infection are widely recognized and their action involves a wide range of processes – from bacterial sensing to triggering innate immune responses. In retaliation to the host immune responses, bacteria target ubiquitin pathways using several virulence factors [reviewed in Ref. (3)]. In this review, we focus on the impact of ubiquitin pathways during infection with *S. enterica* Typhimurium in the context of the innate immune system. We also highlight how studies on the host–pathogen interactions can help to understand the ubiquitination pathway in the eukaryotic cell.

REGULATION OF INNATE IMMUNE SYSTEM IN *SALMONELLA* INFECTION

To successfully colonize the host, the pathogens battle the highly sophisticated defense mechanisms of the innate and adaptive immune systems. Briefly, after ingestion of *Salmonella*, bacteria encounter the harsh acidic environment of the stomach, which they counteract by inducing the acid tolerance response system (4, 5). In the small intestine, *Salmonellae* are awaited by a thick layer of mucus covering the gut epithelium, while the Paneth cells and epithelial cells in the gastrointestinal tract produce antimicrobial peptides that function by disrupting the bacterial cell membrane integrity, which *Salmonella* can counteract. *Salmonella* is able to invade microfold cells of the Peyer's patches and non-phagocytic enterocytes, and the internalized bacteria induce membrane ruffling, which causes formation of *Salmonella*-containing vacuole (SCV), an intracellular niche where the bacteria replicate and thrive with the help of bacterial effectors from the Type III Secretion System [T3SS; reviewed in Ref. (2, 6)]. Another line of host defense includes engulfment of *Salmonella* by macrophages, neutrophils, or dendritic cells, which can lead to phagocytosis. On a molecular level, the innate immune system is activated in response to pathogen-associated molecular patterns (PAMPS), which are conserved components detected on the microbes, such as lipopolysaccharides (LPS), peptidoglycan, or lipoteichoic acid. Since these components are physiologically important for bacterial survival, they cannot be altered as an adaptation strategy. PAMPs are recognized by the germline-encoded pattern recognition receptors (PRRs) of the host cells. PRRs are expressed by non-immune and innate immune cells, and include Toll-like receptors (TLRs), NOD-like receptors (NLRs), and RIG-I-like receptors (RLRs) [reviewed in Ref. (7, 8)]. Signals transduced from the PRRs cause activation of transcription factors, e.g., nuclear factor kappa B (NF-κB), or interferon regulatory factors (IRFs). This leads to expression of key cytokines and chemokines that trigger anti-microbial responses and recruit immune cells to the infected area [reviewed in Ref. (9)]. Immune responses have to be ideally and promptly controlled and, therefore, post-translational modifications (PTMs) of proteins, such as ubiquitination, play here a crucial role.

UBIQUITIN – A SMALL PROTEIN MODIFIER

Ubiquitination is a PTM characterized by the addition of ubiquitin to a lysine residue of protein substrates. It can mark proteins for degradation or play a non-proteolytic role in regulation of processes such as endocytosis, DNA repair, intracellular trafficking,

and signal transduction [reviewed in Ref. (3, 10)]. Ubiquitination is a multi-step process carried out by E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin ligase) enzymes, and it can be reversed by deubiquitinases (deubiquitinating enzymes). Attachment of a single ubiquitin moiety is called monoubiquitination, which can lead to protein autoinhibition, and this has been shown for example in ubiquitin receptors involved in endocytosis [reviewed in Ref. (11)]. Apart from that, ubiquitin can form eight distinct chains, in which the C-terminus of a distal moiety is attached to one of the seven lysine residues of ubiquitin (K6, K11, K27, K29, K33, K48, or K63). These distinct ubiquitin chains have different effects on protein substrates. K63-linked chains can affect cell signaling, receptor endocytosis, or processes associated with DNA repair [reviewed in Ref. (12)], and all other ubiquitin chains target proteins for degradation (13). In addition, the polyubiquitin can be linked through the N-terminal M1, and the chains can also have mixed typology. To add to this complexity, there are ubiquitin-like proteins, such as neural precursor cell expressed, developmentally down-regulated 8 (NEDD8), small ubiquitin-related modifier (SUMO), interferon-induced 17 kDa protein (ISG15), autophagy-related (ATG) 8, or ATG12. Since these PTMs have profound effects on protein function, stability, or localization, it is not surprising that they are employed in host responses to bacterial infections, or that bacterial pathogens evolved complex strategies to interrupt normal cell functions and modify these PTMs to their advantage.

UBIQUITINATION-REGULATED HOST DEFENSE STRATEGIES

SALMONELLA AND UBIQUITIN-REGULATED SELECTIVE AUTOPHAGY

Cells remove unwanted bulk cytosolic materials such as proteins, organelles, or intruding pathogens by autophagy process, which is facilitated by autophagosomes that engulf the cytosolic components and fuse with the lysosomes to form autolysosomes, finally resulting in their degradation. Selective autophagy occurs when the ubiquitin system is used to mark the unnecessary cytosolic materials for degradation via the autophagosomes (3). To selectively bind the ubiquitinated materials, p62, nuclear dot protein 52 kDa (NDP52), and optineurin (OPTN) receptors act as a bridge between the ubiquitinated cargo and the autophagosome (3). *Salmonellae* can be coated with ubiquitin for degradation by the autophagy, but they also developed strategies to escape it. ATG proteins belonging to the ubiquitin or ubiquitin-like families, deubiquitinases, or E3 ligases are described in the sections below (see **Table 1**).

Function of NDP52, p62, and OPTN in autophagy of Salmonella

NDP52 is an autophagy receptor, which is able to detect the ubiquitin moieties on *Salmonella* by using its zinc finger domain. Knockdown of NDP52 leads to enhanced proliferation of bacteria in HeLa cells and to an increase of ubiquitin-coated *Salmonella*. Moreover, NDP52 controls autophagy of *Salmonella* and recruits autophagosomal marker, microtubule-associated protein 1A/1B-light chain 3 [LC3; (14)]. The p62 protein is a ubiquitin-binding protein associated with ubiquitinated protein aggregates that accumulate, for example, in various neurodegenerative disorders (15). In HeLa cells, p62 binds to ubiquitin through its C-terminal ubiquitin-associated (UBA) domain and it also binds to LC3, facilitating autophagy of cytosolic ubiquitin-coated *Salmonella* (16). The autophagy receptor OPTN contains the ubiquitin binding in ABIN and NEMO (UBAN) domain to bind ubiquitin (17) and also binds LC3 through its LIR (LC3-interacting region) motif. OPTN knockdown in HeLa cells during *Salmonella* infection leads to bacterial proliferation. Ubiquitin-binding deficient OPTN mutant or LIR mutant cannot rescue the dysfunction caused by the OPTN knockdown, indicating that both these domains are required to restrict bacterial growth. TANK-binding kinase 1 (Tbk1) phosphorylates OPTN recruited to ubiquitin-coated cytosolic *Salmonella*, thereby enhancing its binding to LC3 and most likely facilitating clearance of cytosolic bacteria through selective autophagy. As mentioned above, there are several *Salmonella*-sensing receptors, including p62, NDP52 as well as OPTN, and all of them bind to ubiquitin-coated *Salmonella*. However, NDP52 and OPTN localize to different microdomains on the surface of ubiquitin-coated *Salmonella* in comparison with p62 (18). This differential recognition might be caused by diverse affinity of these receptors for various ubiquitin linkages or by secondary interactions with other proteins.

Table 1 | Host proteins relevant in ubiquitin-mediated response to *Salmonella* infection.

Host protein	Function	Physiological effect	Reference
OPTN	Contains ubiquitin-binding domain; autophagy receptor	Selective autophagy of ubiquitin-coated *Salmonella*	(18)
p62	Autophagy receptor	Autophagy of ubiquitin-coated *Salmonella*	(15, 16)
NDP52	Autophagy receptor	Autophagy of ubiquitin-coated *Salmonella*	(14)
LRSAM1	RING-type E3 ligase	Restriction of bacterial replication, required for autophagy of *Salmonella*	(19)
USP18	ISG15-specific deubiquitinase	Regulation of inflammatory response to *Salmonella*, IFN signaling	(25)
UCH-L1	Deubiquitinase	Increase in bacterial uptake, remodeling of actin cytoskeleton	(26)
HsRMA1	E3 ligase	Ubiquitination of bacterial SopA, induces bacterial escape to cytosol from SCV	(28)
UbcH5c	E2 enzyme	SopB localization to SCV, works with TRAF6	(29)
TRAF6	RING-type E3 ligase	Ubiquitination of bacterial effector SopB, downregulation of SopB activity and its localization in SCV	(29–31)
TRIM21	E3 ligase	Recognition of intracellular antibodies during infection	(27)

Host E3 ligase LRSAM1

In a study dissecting the function of autophagy cascade in elimination of *Salmonella*, leucine-rich repeat (LRR) and sterile alpha motif-containing protein 1 (LRSAM1) has been identified as an E3 ubiquitin ligase responsible for recognition and ubiquitination of *Salmonella* and its subsequent autophagy. LRSAM1 co-localizes with *Salmonella*, and a knockdown of LRSAM1 results in increased replication of bacteria in the cytoplasm of HeLa cells. Co-localization of LRSAM1 with *Salmonella* was also observed in infected murine bone marrow-derived macrophages and intraperitoneal macrophages. LRSAM1 contains a domain commonly found in innate PRRs, LRR, as well as RING domain, which is characteristic of one of the classes of ubiquitin E3 ligases. LRR is required and sufficient for the LRSAM1 localization to *Salmonella*, but RING domain is essential for its ubiquitination. LRSAM1 and previously mentioned NDP52 localize to intracellular bacteria into spatially separated subdomains, but NDP52 recruitment to *Salmonella* is dependent on LRSAM1, which is also required for ubiquitin-associated autophagy and most likely can recognize bacteria by itself. Moreover, polyubiquitination directed by LRSAM1 favors K6- and K27-conjugated ubiquitin chains in comparison to other linkages. LRSAM1, therefore, restricts bacterial replication in the cytoplasm and is crucial for ubiquitin-mediated autophagy (19). This study helped to identify mechanisms and specificity of a novel host ubiquitin E3 ligase and define its function in autophagy. Selective autophagy during *Salmonella* infection is not completely understood; yet, it is clear that it requires ubiquitin pathways to function efficiently and it represents an effective host surveillance mechanism to control *Salmonella* replication and prevent systemic infection.

DEUBIQUITINATION IS RELEVANT IN INFLAMMASOME ASSEMBLY DURING *SALMONELLA* INFECTION

Inflammasome includes PRRs such as NLRs, which are assembled into a multiprotein complex that activates caspase-1 and leads to secretion of proinflammatory interleukins (IL), such as IL-1β, which can lead to pyroptosis, a proinflammatory cell death [reviewed in Ref. (20)]. Role of ubiquitination was investigated in LPS- and *Salmonella*-induced inflammasome. Treatment with the general deubiquitinase inhibitors (PR-619 and WP1130) led to increase in polyubiquitination of NLRP3 (NLR family, pyrin domain containing 3) in N1-8 macrophages stimulated with LPS and ATP. These inhibitors also interfered with caspase-1 activation during *Salmonella* infection (21). This suggests that deubiquitinases are involved in the inflammasome function. Moreover, treatment of cells with b-AP15, which inhibits ubiquitin-specific peptidase 14 (USP14) and ubiquitin carboxy-terminal hydrolase 37 (UCH37), caused inhibition of ATP-, or nigericin- induced IL-1β release from LPS-primed macrophages. Deubiquitinase inhibition also led to impairment in apoptosis-associated speck-like protein containing CARD (ASC) oligomerization without direct inhibition of caspase-1 activity. This has not been shown directly in *Salmonella* infection model and it is not known how these deubiquitinases affect the infection outcome (22). These studies were crucial in identification of a novel mechanism of inflammasome regulation by deubiquitinases.

ISG15-SPECIFIC PROTEASE IMPORTANT IN INTERFERON SIGNALING IN *SALMONELLA* INFECTION

Interferon-induced 17 kDa protein (ISG15) post-translationally modifies other proteins and its expression is induced by type I interferons (IFN) or by exposure of cells to LPS (23). One of the proteins that removes ISG15 modification is ubiquitin-specific peptidase 18 [USP18; (24)]. A mutation in USP18 leads to an increased bacterial load in spleen and liver in mice, and it is also associated with an altered inflammatory response to *Salmonella* infection, e.g., increase in Type 1 IFN or IL-6 secretion, but a decrease in STAT4 phosphorylation and IFN-γ production (25). This suggests that this ISG15-specific deubiquitinase is required for host resistance to *Salmonella* infection by contributing to the IFN signaling, which might also be relevant in other infections.

UCH-L1 PROMOTES UPTAKE OF *SALMONELLA* IN EPITHELIAL CELLS

Ubiquitin C-terminal esterase L1 (UCH-L1) is a deubiquitinase promoting the invasion of cells by *S. enterica* and *Listeria monocytogenes*. The internalization of bacteria by epithelial cells was significantly decreased in UCH-L1 knockdown cells, while the overexpression of UCH-L1 leads to an increased uptake of bacteria. The mechanism, by which this enzyme regulates bacterial uptake possibly involves the actin cytoskeleton remodeling, since overexpression of UCH-L1 was associated with an increase in formation of the actin stress fibers, while overexpression of catalytically inactive C90S mutant of UCH-L1 had an opposite effect (26). This study identified new functions of UCH-L1 in host cells.

FUNCTION OF E3 UBIQUITIN-PROTEIN LIGASE TRIM21 IN IMMUNE SIGNALING

E3 ubiquitin-protein ligase tripartite motif containing 21 (TRIM21) is a cytosolic antibody receptor that recognizes intracellular antibodies during infection. TRIM21 catalyzes the formation of K63-linked polyubiquitin chains and leads to stimulation of the NF-κB, AP-1, IRF3, IRF5, and IRF7 pathways. During infection of HeLa cells by *Salmonella*, antibodies are carried into the cell by the bacteria. TRIM21 E3 ligase co-localizes to a portion of antibody-bound bacteria. Moreover, antibody-dependent NF-κB signaling is hindered when TRIM21 is knocked down. This study emphasized another general aspect of involvement of ubiquitin in immune signaling (27).

EXPLOITATION OF THE HOST RESPONSES BY *SALMONELLA*-ENCODED PROTEINS IN THE CONTEXT OF UBIQUITIN SIGNALING

Salmonella has evolved several defense strategies to survive the hostile environment of the host cell. Since ubiquitin pathway is extensively used by the immune system, bacteria strategically exploit it via their effector proteins. First, SseL and AvrA are deubiquitinases encoded by *Salmonella*, which function by preventing autophagy and inflammatory responses, respectively (**Table 2**). Second, *Salmonella* effectors SopA, SspH1, SspH2 and Slrp are ubiquitin E3 ligases, which ubiquitinate protein substrates and some are even capable of auto-ubiquitination (**Table 2**). Third, bacteria take advantage of the host E3 ligases to add ubiquitin moieties to their own proteins, such as SopA, SopB, SopE, or SptP

Table 2 | Bacterial proteins relevant in ubiquitin-mediated response to *Salmonella* infection.

Bacterial protein	Activity	Substrates	Physiological effect	Host cell type studied	References
SseL	Deubiquitinase	Ubiquitinated aggregates, ALIS	Delayed cytotoxic effect in macrophages, prevention of autophagy	J774 and RAW264.7 macrophages, murine bone marrow-derived macrophages, HeLa	(33–35)
AvrA	Deubiquitinase	IκBα, β-catenin	Inhibition of NF-κB pathway	*In vivo* (mouse), HCT116, HEK293, HeLa	(43)
SopA	E3 ligase	–	–		(46)
SspH2	E3 ligase	Nod1	Modulates innate immunity in host cells by increasing the Nod1-mediated IL-8 secretion	HeLa and HEK293T	(48)
SspH1	E3 ligase	PKN1	Attenuates androgen receptor signaling	HEK293	(52)
Slrp	E3 ligase	Trx	Triggers cell death	HeLa	(54)

(**Table 1**). The exploitation of the host responses by *Salmonella*-encoded proteins in the context of ubiquitin signaling is described in the sections below.

SALMONELLA DEUBIQUITINASES

Salmonella deubiquitinase SseL interferes with autophagy

SseL is *Salmonella*'s effector protein that functions as a deubiquitinase. SseL prevents the autophagy machinery from recognizing ubiquitin aggregates and aggresome-like induced structures (ALIS), which are formed in response to bacterial infection or LPS-treatment (32, 33). Infection with SseL-deficient *Salmonella* strain results in an accumulation of SCV-associated ubiquitinated aggregates in HeLa cells compared to cells infected with wild-type *Salmonella*. Moreover, by deubiquitination of these ubiquitinated aggregates SseL decreases autophagic flux in macrophages and it favors intracellular replication of *Salmonella* in bone marrow-derived macrophages (33). Ubiquitin-driven autophagy was identified as one of the host responses to *Salmonella* and SseL is an effector protein that counteracts this process. Additionally, SseL is necessary for bacterial virulence in mice, required for delayed cytotoxicity by *Salmonella* in macrophages (34, 35), and it binds to the oxysterol-binding protein [OSBP; (36)]. The deubiquitinating activity of SseL is also related to its role in cell lipid metabolism as SseL prevents lipid droplet accumulation in mouse epithelial cells (37). Since lipid droplet metabolism is regulated in autophagy (38), it could potentially be related to SseL's function in autophagic flux, although the function of SseL appears to be complex and it might involve several substrates and pathways. From the studies on SseL, some more general host mechanisms could be identified, such as the ubiquitin involvement in the selective autophagy.

NF-κB pathway modulation by Salmonella deubiquitinase AvrA

NF-κB is a conserved family of transcription factors that regulate diverse processes, such as inflammation, immune response, cell growth, and apoptosis [reviewed in Ref. (39, 40)]. Although induction of this pathway provides immediate immune response and host protection, pathogens utilize the immune cells to replicate and spread to other tissues in the host, resulting in systemic infection. For example, infection with the virulent *Salmonella* leads to increased inflammatory response by NF-κB pathway activation, while the avirulent strain has an opposite effect (41). Inhibition of the NF-κB pathway is facilitated by AvrA, which is another *Salmonella*-encoded deubiquitinase (42, 43) that also functions as an acetyltransferase (44). AvrA deubiquitinates and therefore stabilizes IκBα, an inhibitor of NF-κB pathway, thus preventing nuclear translocation of NF-κB p65, which was shown *in vivo* in mice and in epithelial cells. Infection of mice with AvrA-deficient strain of *Salmonella* leads to an increased IκBα degradation and secretion of NF-κB dependent cytokine, IL-6. AvrA also stabilizes an inhibitor of the proinflammatory NF-κB pathway, β-catenin, by preventing its proteasomal degradation via removal of ubiquitin moieties from β-catenin (43). Moreover, AvrA was linked to an increased risk of cancer associated with chronic *Salmonella* infections (45). In summary, AvrA is a bacterial effector protein used to fight the host defense strategies marked by the ubiquitin modification.

SALMONELLA-ENCODED UBIQUITIN E3 LIGASES

SopA E3 ligase controls effective bacterial escape into the cytosol

Salmonella's effector, SopA, is a HECT-like E3 ubiquitin ligase that becomes ubiquitinated by a host E3 ligase HsRMA1 (28, 46), although it is also capable of autoubiquitination (47). In an ubiquitination assay to identify the E2 ligases associated with SopA, UbcH5a, UbcH5c, and UbcH7 were preferentially used by SopA, suggesting a regulatory role in inflammation (46). We discuss effects of SopA polyubiquitination in Section "SopA polyubiquitination regulating bacterial escape."

SspH2 functions in innate immune responses

Salmonella's SspH2 belongs to the novel ubiquitin E3 ligase (NEL) family. It contains LRR domain, which exerts an inhibitory effect on the NEL domain activity, while the NEL domain expressed alone has a 25-fold increase in E3 ligase activity in comparison to a full-length SspH2. Moreover, in epithelial cells, SspH2 increases the Nod1-mediated IL-8 secretion via monoubiquitination, thereby mediating the innate immune response. This function depends on the E3 ubiquitin ligase activity of SspH2

(48). Apart from the identification of a novel bacterial E3 ligase, these studies bring into light a new modification of the host Nod1 protein and its relevance in the IL-8 secretion.

Role of SspH1 in androgen receptor signaling

SspH1 is *Salmonella*'s effector protein that is a member of the NEL family of ubiquitin E3 ligases (49). It is capable of ubiquitination of protein kinase N1 (PKN1), which functions in androgen receptor (AR) signaling (50, 51). The wild-type SspH1 ubiquitinates PKN1 when co-expressed in HEK293 cells and targets it to the 26S proteasome for degradation, but a catalytic mutant of SspH1 lacks this function. Attenuation of AR activation was observed when wild-type SspH1 was transiently expressed in HEK293 cells in comparison to expression of C492A catalytic mutant or PKN1-interaction mutant. By mediation of ubiquitination and subsequent degradation of PKN1, SspH1 attenuates AR signaling, which might be important in regulation of the cellular immunity during *Salmonella* infection (52, 53).

Slrp ubiquitinates Trx and has cytotoxic effect on host cells

Slrp is another *Salmonella*'s ubiquitin E3 ligase, which belongs to NEL E3 ligase family, and it also contains LRR domains. Mammalian thioredoxin-1 (Trx) is as a binding partner of Slrp (54). Trx proteins regulate redox-related signaling, synthesis of cytokines, growth, and apoptosis [reviewed in Ref. (55, 56)]. Slrp ubiquitinates Trx, while mutation in the cysteine active site of Slrp (C546) disables this activity. In HeLa cells, Slrp expression results in reduction of Trx's activity in confluent but not in growing cultures. *Salmonella* infection of HeLa cells decreases Trx activity, which also correlates with an increase in cell death. Collectively, these findings suggest that the E3 ligase activity of Slrp is partially responsible for the cytotoxic effect on HeLa cells during infection (54).

UBIQUITINATION OF BACTERIAL EFFECTOR PROTEINS

Salmonella's effector proteins, SopA, SopB, SopE, and SptP, have been all shown to be ubiquitinated, and in some cases, it marks them for proteasome-dependent degradation [e.g., SopE and SptP; (57)]. SopA and SopB ubiquitination is relatively well understood and it is described below.

SopA polyubiquitination regulating bacterial escape

SopA is an ubiquitin E3 ligase that can be ubiquitinated by a host E3 enzyme HsRMA1 (28, 46). SopA ubiquitination by HsRMA1 is important in regulation of the bacterial escape from SCV, as shown by using HsRMA1 knockdown study. Also, compared to the wild-type *Salmonella*, a *sopA Salmonella* mutant has an impaired ability to escape from SCV into the cytosol in HeLa cells. This together demonstrates that ubiquitination of this effector protein is important for an effective bacterial escape into the cytosol and that host E3 ligase HsRMA1 contributes to this function (28).

Function of SopB polyubiquitination affects its activity and intracellular localization

Salmonella's SopB, is a phosphoinositide phosphatase that regulates several physiological processes owing to its phosphatase activity [reviewed in Ref. (58)]. It is ubiquitinated by host E3 ligase TRAF6 and E2 enzyme UbcH5c (29). Interestingly, SopB ubiquitination does not affect SopB protein stability but it downregulates SopB activity at the plasma membrane. SopB ubiquitination also leads to an internalization of SopB into the host cells, and it causes retention of SopB in the SCV (31). Ubiquitination of SopB is essential for SopB-dependent recruitment of Rab5 to SCV (30), but not for PI(3)P generation on the SCV (31). In summary, SopB ubiquitination by the host ubiquitin machinery is not related to its stability but it does regulate its enzymatic activity at the plasma membrane as well as its intracellular localization.

CONCLUSION

Ubiquitination is a widespread PTM critical in regulation of many host cellular pathways. However, due to the expansive involvement of this modification in cellular processes, a lot has to be learnt about the function and mechanisms of ubiquitination. Since *Salmonella* infection is important from the human health point of view, there are many efforts concentrated on dissecting the cellular responses to this bacterial infection. In particular, the involvement of ubiquitination in the host–pathogen interactions during *Salmonella* infection is extensive. Due to the work on ubiquitin pathways in *Salmonella* infection, functions and substrates of such deubiquitinating enzymes as USP18 (25) and UCH-L1 (26) were identified. Similarly, more functions were discovered of the host ubiquitin E3 ligases, such as HsRMA1 (28), LRSAM1 (19), and TRAF6, and about host ubiquitin E2 enzyme, UbcH5c (29). Furthermore, the work focused on autophagic clearance of *Salmonella* has been critical in identification of novel mechanisms controlling the autophagy receptors. Specifically, TBK1-mediated phosphorylation of a receptor OPTN leads to selective autophagy of ubiquitin-coated *Salmonella*. This can constitute a more universal mechanism for selective autophagy (18). In fact, TBK1-mediated phosphorylation of OPTN has been recently shown to regulate autophagic clearance, which is relevant in autophagy-mediated degradation of misfolded protein inclusions, for example, in some neurodegenerative disorders (59). All these examples highlight how studies on *Salmonella* infection can lead to characterization of general mechanisms in host cells, and to a better understanding of ubiquitin enzymes that have physiological roles beyond the responses to the bacterial infection.

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Salmonella–host interactions – modulation of the host innate immune system

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Salmonella enterica (*S. enterica*) are Gram-negative bacteria that can invade a broad range of hosts causing both acute and chronic infections. This phenotype is related to its ability to replicate and persist within non-phagocytic host epithelial cells as well as phagocytic dendritic cells and macrophages of the innate immune system. Infection with *S. enterica* manifests itself through a broad range of clinical symptoms and can result in asymptomatic carriage, gastroenteritis, systemic disease such as typhoid fever and in severe cases, death (1). Exposure to *S. enterica* serovars Typhi and Paratyphi exhibits clinical symptoms including diarrhea, fatigue, fever, and temperature fluctuations. Other serovars such as the non-typhoidal *Salmonella* (NTS), of which there are over 2,500, are commonly contracted as, but not limited to, food-borne sources causing gastrointestinal symptoms, which include diarrhea and vomiting. The availability of complete genome sequences for many *S. enterica* serovars has facilitated research into the genetic determinants of virulence for this pathogen. This work has led to the identification of important bacterial components, including flagella, type III secretion systems, lipopolysaccharides, and *Salmonella* pathogenicity islands, all of which support the intracellular life cycle of *S. enterica*. Studies focusing on the host–pathogen interaction have provided insights into receptor activation of the innate immune system. Therefore, characterizing the host–*S. enterica* interaction is critical to understand the pathogenicity of the bacteria in a clinically relevant context. This review outlines salmonellosis and the clinical manifestations between typhoidal and NTS infections as well as discussing the host immune response to infection and the models that are being used to elucidate the mechanisms involved in *Salmonella* pathogenicity.

Keywords: gastroenteritis, host innate immunity, macrophages, NTS, pathogenicity islands, salmonellosis

INTRODUCTION

Every year, thousands of cases of salmonellosis are reported worldwide. However, the actual number of infections may be very different and many times greater than expected since many milder cases are not diagnosed or reported (http://www.cdc.gov/salmonella). *Salmonella* infection or the disease associated with it, salmonellosis, is most often characterized by enteritis. However, host restricted serotypes tend to induce higher levels of bacteremia, while some human restricted serotypes cause a systemic disease that is characterized by mild symptoms (2). Children are the most likely group of individuals to present salmonellosis. The rate of diagnosed infections in children <5 years old is higher than the rate diagnosed in all other persons. Other groups of risk, such as the elderly and immunocompromised individuals are the most likely to present severe forms of the disease.

Persons with diarrhea usually recover completely after a few days of the initial infection, although it may be several months before their bowel habits return to normal. Contrary to this could be a small number of persons with *Salmonella* infections that develop pain in their joints, irritation of the eyes, and painful urination. Taken together, these symptoms indicate a disease called reactive arthritis. This disease can last for months or years, and can lead to chronic arthritis, which is extremely difficult to treat. Antibiotic treatment does not make a difference in whether or not the person develops arthritis (3). Other types of invasive infections caused by *Salmonella*, such as bacteremia, osteomyelitis, and meningitis, may also occur and in these cases may require antimicrobial therapy (4).

The continuous evolution of *Salmonella* at the genetic and genomic levels contributes to the increased virulence and resistance to multiple antibiotics, leading to a phenotype of multidrug resistance. This resistance is a significant public health concern (5). Two major changes in the epidemiology of non-typhoidal salmonellosis have occurred in the last century. These were the emergence of food-borne human infections caused by *Salmonella enterica* Enteriditis and by multidrug-resistant strains of *Salmonella enterica* Typhimurium. In this century, a concerning situation is the increased resistance that non-typhoidal *Salmonella* (NTS) presents to fluoroquinolones and third-generation cephalosporins. Clinical isolates showing carbapenem resistance have also being reported (4). In terms of therapy, treatment with antibiotics is not usually recommended for uncomplicated *Salmonella* gastroenteritis. However, recent studies indicated that a 3–5 days therapy with ceftriaxone for patients with severe

gastroenteritis could lead to a faster recovery. A continuous surveillance scheme of *Salmonella* infections in both humans and animals is of importance. A better understanding of the mechanisms that can lead to the emergence of antimicrobial resistance in *Salmonella* may help develop better interventional strategies that can ultimately reduce the spread of resistant *Salmonella* between humans and reservoirs identified (or not) along the food chain.

Due to the importance of *Salmonella* in the clinical and public health setting, there has been a significant effort to deepen the knowledge about pathogenic determinants of this bacterium. The clinical relevance of the disease, associated with the advances on the molecular tools available to study *Salmonella* and the development of suitable animal models, have lead to the development of optimal conditions to drive the scientific community to generate a large expansion of our knowledge about the pathogenesis of *Salmonella*-induced enterocolitis (6). This research effort has also generated an increased amount of information on the host immune mechanisms that complements gaps that still exist in fundamental research developed in this area.

The goal of this review is to discuss salmonellosis, the clinical signs caused by *Salmonella* infections, and the advances in our knowledge on the innate intestinal immunity. Additionally, the interaction with the host and the models used to elucidate the mechanisms triggered by the interaction of *Salmonella* with the host will also be discussed.

INTERACTIONS OF *SALMONELLA* WITH THE GUT MICROBIOME

The intestinal microbiome, which is host to an estimated 1×10^{14} bacteria, is responsible for conferring numerous aspects of the host response against salmonellosis (7). As many as 1,000 species of bacteria inhabit this niche, with the majority being classified as Gram-positive Actinobacteria and Firmicutes as well as Gram-negative bacteroides (8). A healthy gut microbiome provides protection against epithelial cell invasion via a series of strategies including the production of toxic metabolites, which have been shown to repress the expression of *Salmonella* virulence genes among others. This feature assists in the clearance of pathogens from the gut lumen after NTS-induced diarrhea (7). Increased fecal shedding and establishment of carrier status is commonly associated with prolonged treatment with antimicrobial compounds as these can have adverse effects on the composition of the gut microbiome of an individual (8, 9). This depletion of the natural gut microbiome may have long lasting effects and can result in an increased susceptibility to *Salmonella* colonization. One such example of this scenario is *S.* Typhimurium, which takes advantage of the availability of ethanolamine, a nutrient present in the microbiome, to gain a significant growth advantage in the intestine during inflammation over potential competing pathogens. *S.* Typhimurium-encoded virulence factors have been shown to induce the production of an alternate electron acceptor by the host, which supports anaerobic respiration and enables *S.* Typhimurium to outcompete other fermenting gut microbes sharing the same ecological niche (10).

SALMONELLOSIS

Salmonellosis causes significant morbidity and mortality on a global scale and occurs after the ingestion of food or water sources that have been previously contaminated by the fecal or urinary excretions of animals that can act as reservoirs of *Salmonella* (11). Following infection with *Salmonella* species, a broad range of clinical manifestations can be presented in a number of ways depending on the susceptibility of the host (12, 13). These include bacteremia, enteric fever, enterocolitis, and chronic asymptomatic carriage. Typhoid and Paratyphoid fever, collectively termed enteric fever, are contracted following infection with *S. enterica* serovars Typhi (*S.* Typhi) and Paratyphi (*S.* Paratyphi), respectively. In contrast, gastroenteritis is commonly associated with NTS serovars such as Typhimurium (*S.* Typhimurium) and Enteritidis (*S.* Enteritidis).

In human beings, *S.* Typhi and *S.* Paratyphi cause typhoid fever, a bacteremic illness, which presents in a unique manner when compared with other Gram-negative bacteremias (14, 15). *S.* Typhi has previously adapted to infect human hosts whereas other serovars have retained a broad host preference and are capable of infecting a range of animals causing enterocolitis (16). Serovars of *S. enterica* including Choleraesuis (*S.* Choleraesuis), Dublin (*S.* Dublin), and *S.* Typhimurium can successfully infect both human and animal hosts. However, the infection presents differently in each. Human infection with *S.* Choleraesuis and *S.* Dublin commonly results in bacteremia. In mice, *S.* Typhimurium causes symptoms similar to human typhoid fever and will disseminate throughout the body of the host causing systemic illness (17, 18). Systemic infection can result in a diverse range of clinical manifestations that include bradycardia, hepatomegaly, and splenomegaly. Bacterial emboli form skin lesions known as Rose spots that occur in approximately 30% of typhoid fever cases. NTS serovars cause self-limiting diarrhea and in rare cases, secondary bacteremia. Primary NTS bacteremia has also been reported in immunocompromised hosts (19, 20). Death from salmonellosis can be caused by perforation of the gut and necrosis of Peyer's patches leading to peritonitis or toxic encephalopathy [H. (15)].

SALMONELLA SPECIES AND SUBSPECIES

Salmonella enterica are Gram-negative facultative intracellular anaerobes that can invade a broad range of hosts causing both acute and chronic infections by means of their ability to replicate and persist within non-phagocytic epithelial cells as well as phagocytic dendritic cells and macrophages of the host innate immune system (21, 22). The genus *Salmonella* comprises two species, *S. enterica* and *S. bongori* (also referred to as *subsp.* V). The former is further divided into six subspecies (as shown in **Figure 1**), which are biochemically differentiated into serovars based on the composition of their carbohydrate, flagellar, and lipopolysaccharide (LPS) structures. All *Salmonella* serotypes can be designated by an antigenic formula based on somatic (O) and flagellar (H) antigens in addition to capsular (Vi) antigens (16).

SALMONELLA PATHOGENICITY ISLANDS

Using *ex vivo* and *in vivo* animal models of infection, many virulence factors have been determined, which are responsible for inducing an inflammatory immune response in the infected host. There are two broad categories of proinflammatory stimuli that can be observed during *Salmonella* infection. These are pathogen-associated factors that stimulate the innate immune system of the

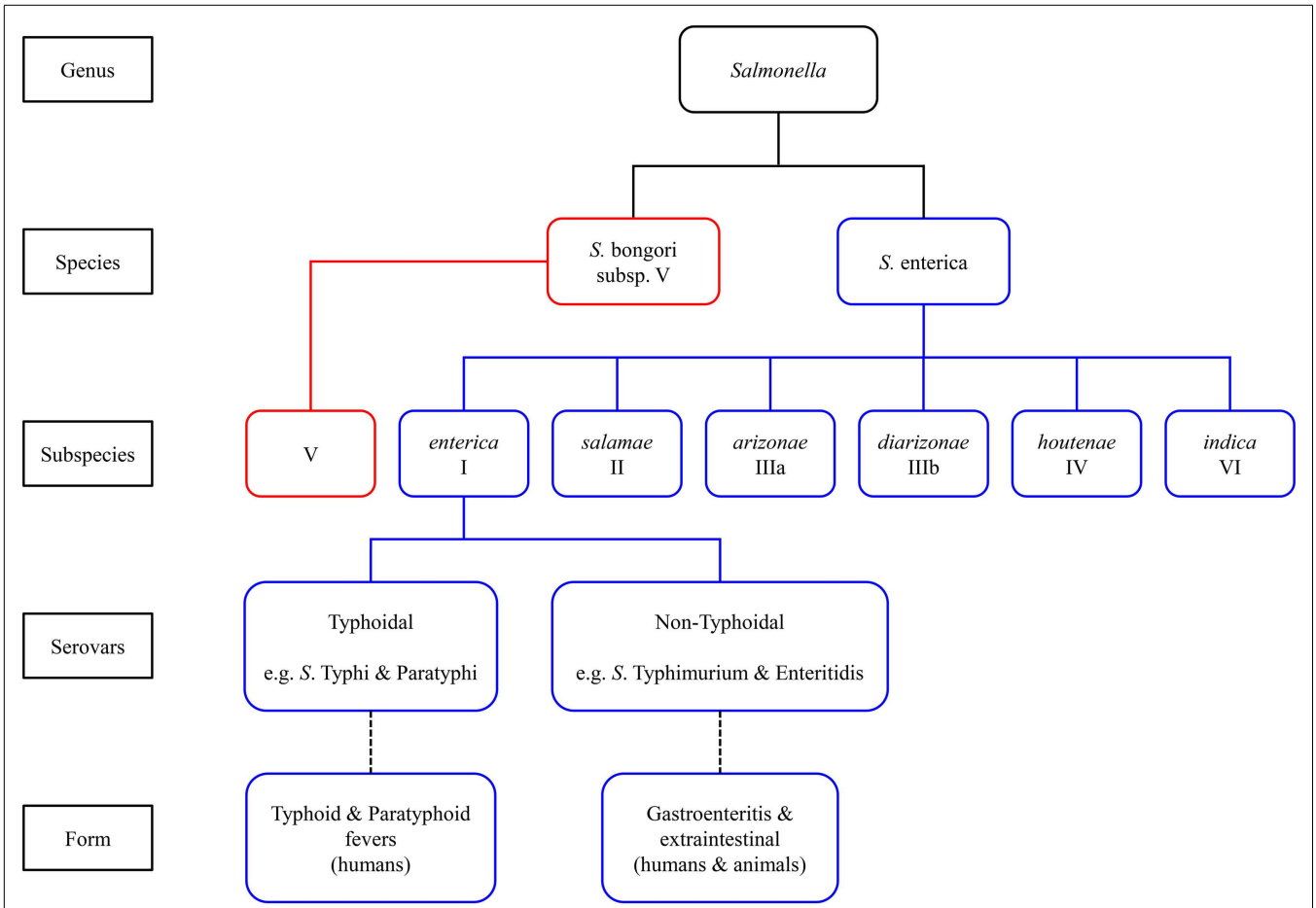

FIGURE 1 | Classification of *Salmonella* species and subspecies.

host and virulence associated factors that exploit host processes resulting in disease pathology.

Salmonella pathogenicity islands (SPI), historically acquired through horizontal gene transfer events, include clusters of genes, which encode the mechanisms through which *Salmonella* acts as a virulent pathogen (23, 24). These genetic islands are located on the bacterial chromosome or on plasmids, however, not all serovars possess every known SPI. SPI-1 through SPI-5 are common among all *S. enterica* serovars (**Table 1**). To date, 23 SPI have been described although the functions of those genes contained within each island have not yet been completely elucidated (25, 26). SPI-1 and SPI-2 are of particular importance in *in vivo* infection (as shown in **Table 1**; **Figure 2**). The SPI encode effector proteins that are translocated directly into host cells across the plasma membrane type III secretion systems (T3SS-1 and T3SS-2) that provide *Salmonella* with the biochemical machinery to exploit this intracellular niche. T3SSs can also be used to secrete effector proteins into the surrounding environment to influence host cell physiology (27, 28) (**Table 1**).

Salmonella pathogenicity islands-1 was originally thought to be important as an invasion-related cluster of genes required for oral virulence (39). More recently, additional functions have been described for this locus. SPI-1-induced activation of the host innate immune system results in inflammation and the recruitment of polymorphonuclear (PMN) cells across the intestinal epithelial barrier following the secretion of the effector protein SipA by *Salmonella*. The latter protein is required in conjunction with the cytokine, IL-8, and pathogen-elicited epithelial chemoattractant (PEEC) to recruit neutrophils as has been reported in cultured epithelial monolayers (40). The production of PEEC can be induced by SipA secretion or by direct addition of SipA to cultured intestinal epithelial monolayers leading to the recruitment of basolateral neutrophils to the apical epithelial membrane (41, 42). SPI-1 effector secretion also leads to NF-κB signaling- and caspase-1-mediated IL-1β/IL-18 activation (43). SipB, an SPI-1 encoded effector protein, which is translocated across the host cell membrane by T3SS-1, is critical for inflammatory disease *in vivo* (38) and is responsible for pyroptotic cell death, a rapid form of programed cell death associated with antimicrobial responses during inflammation that possesses both apoptotic and necrotic features (44, 45). SipB binds caspase-1 (IL-1β converting enzyme) in the cell cytosol resulting in the maturation of proinflammatory cytokines IL-1β and IL-18 into active peptides (46). Further studies have revealed that both caspase-1 and Ipaf deficient mice exhibit

Table 1 | Features and functions of SPI-1 through SPI-5 identified among all *S. enterica* serovars.

Pathogenicity Island	Approximate size (kb)	Type secretion system	Features/functions	Reference
SPI-1	40	Type III secretion system (T3SS)	Invasion of intestinal epithelium; development of SCV; encodes effector proteins important for: actin cytoskeleton rearrangements; membrane ruffling; induce IL-8 and pathogen-elicited epithelial chemoattractant secretion	Ehrbar et al. (29), Hapfelmeier et al. (30); Ibarra et al. (31)
SPI-2	40	Type III secretion system (T3SS)	Survival within phagocytic cells such as macrophage; inhibits fusions between lysosomes and SCVs; endocytic trafficking inhibition; avoidance of NADPH oxidase-dependant killing by macrophages; encodes effector proteins: SpiC, SseF, SseG; encodes chaperone proteins: SscA, SscB, SseA; encodes translocon proteins SseB, SseC, and SseD	Waterman and Holden (32), Hapfelmeier et al. (30), Figueira et al. (33)
SPI-3	17		Intramacrophage survival; encodes macrophage survival protein MgtC; encodes Mg^{2+} transporter MgtB	Blanc-Potard and Solomon (34), Fierer and Guiney (16), Rychlik et al. (35)
SPI4	27	Type 1 secretion system (T1SS)	Mediates adhesion to epithelial cells; encodes genes *siiA-F* (*Salmonella* intestinal infection genes) and SiiE ~600 kDa non-fimbrial adhesion protein; role in oral virulence	Kiss et al. (36), Gerlach et al. (37), Rychlik et al. (35)
SPI-5	8		Encodes SopB (secreted by T3SS of SPI-1); encodes PipB (translocated by T3SS of SPI-2 to the SCV); important for *S.* Dublin virulence and induction of proinflammatory immune response in cattle	Zhang et al. (38), Rychlik et al. (35), Sabbagh et al. (25)

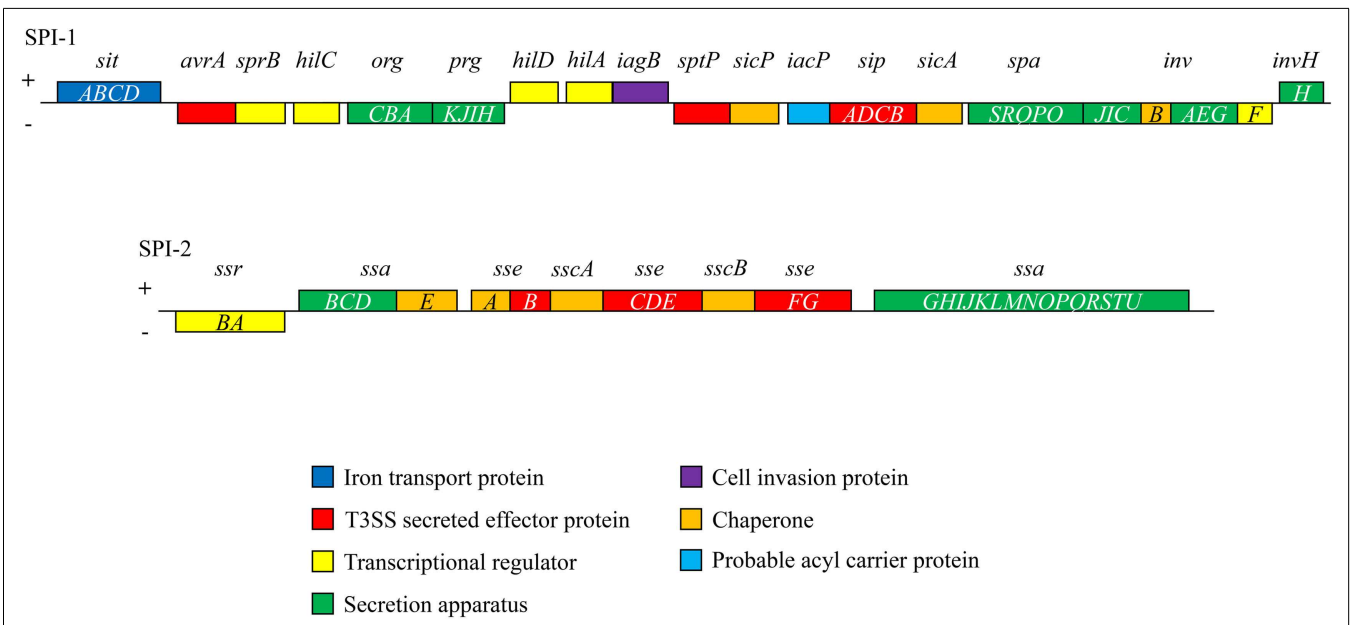

FIGURE 2 | Schematic illustration of the genes of SPI-1 and SPI-2 indicating their functional categories is shown. In *Salmonella*, SPI-1 and SPI-2 encode a range of effector proteins, secretion apparatus, and transcriptional regulators in addition to T3SS-1 and T3SS-2.

an increased susceptibility to typhoid fever, thereby demonstrating the protective proinflammatory role played by caspase-1 (47).

The proinflammatory activity of SPI-2 while less characterized has been shown to be important for intracellular persistence and systemic virulence in murine typhoid fever in addition to evading host phagosome oxidation mechanisms (48). T3SS-2 plays an important role in inflammatory disease, highlighting the involvement of SPI-2 in the onset of enterocolitis. SPI-2 functions by enabling the translocation of effectors across the membrane of the *Salmonella*-containing vacuole (SCV) in infected host cells.

The genes encoding T3SS-2 are controlled by two-component regulatory systems such as OmpR–EnvZ and the SPI-2 encoded SsrA–SsrB. As many as 28 SPI-2 encoded effectors have been identified to date with many of these currently of unknown function such as SseK1-3 and SteA–B, D–E. SseF is involved in SCV localization and *Salmonella*-induced filament (Sif) formation. PipB2 is responsible for kinesin-1 recruitment to the SCV and Sif extension, whereas SspH2 and SteC are recruited to and involved in the formation of the SCV-associated F-actin meshwork, respectively (49). The Toll-like receptors (TLR) adapter, myeloid differentiation primary response gene (MyD88) is required for SPI-1 independent intestinal inflammation in mice (30).

THE INTERACTION OF *SALMONELLA* WITH THE HOST

Salmonella invades both phagocytic and non-phagocytic cells including mononuclear phagocytic cells present in the lymphoid follicles, liver, and spleen. Epithelial cells and phagocytic cells such as dendritic cells, neutrophils, and macrophages identify specific pathogen-associated molecular pattern (PAMP) motifs and endogenous danger-associated molecular pattern molecules (DAMPs) present in the bacteria. Pattern-recognition receptors (PRRs), which include NOD-like receptors (NLRs) and TLRs, comprise the early components of the immune system that function to detect invading pathogens through PAMPs and DAMPs and signal to recruit and activate phagocytic cells such as neutrophils and macrophages (50, 51). These receptors trigger an immune response and are key to establishing an important network between the innate and adaptive immune systems. Bacterial DNA, flagella, and LPS are examples of PAMPs, which activate TLR4, TLR5, and TLR9 signaling in the host. LPS-induced TLR4 activation is important for triggering the inflammatory responses of the host. It also plays an important role in mounting an inflammatory response to intravenously administered LPS. Mice with mutations in TLR4-encoding genes exhibit an increased susceptibility to *Salmonella* infection irrespective of other *Salmonella* resistance *loci* (52, 53). Additionally, LPS plays an important role in the onset of sepsis during systemic infection as observed by its role in inducing inflammation in macrophages (54).

The immune system can be divided into two main parts: the innate or non-specific and the adaptive or specific components. The innate immune system is the first host challenge presented to invading pathogens whereas the adaptive immune system provides further protection in addition to an immunological memory, which enables a faster response upon repeat exposure to the same pathogen or antigen. In addition to cellular components such as phagocytic cells, there are humoral elements such as the complement system that make up the innate immune system. Additionally, anatomical features like the mammalian skin layer act as physical barriers to infection. The interplay between the innate and adaptive immune systems, including different types of cells and molecules such as cytokines and antibodies, form the totality of the host immunity.

Leukocytes of the innate immune system include phagocytic cells, namely dendritic cells, macrophages, and neutrophils, which can engulf foreign antigens, particles, or pathogens. These phagocytic cells are recruited following the release of specific cytokine signals. These cells serve an important role in the activation of the adaptive immunity, which usually assumes the presence of lymphocytes (55). Other cells, such as basophils, eosinophils, and mast cells are also part of the host innate immune system that contributes to the innate immunity.

During the initial stages of an inflammatory response, neutrophils and macrophages are recruited to the site of infection. Neutrophils phagocytose the invading pathogens and kill them intracellularly. Similarly, macrophages and newly recruited monocytes, which will differentiate into macrophages following signaling or chemical stimulation, also function by phagocytosing and killing the pathogens at the intracellular level. Furthermore, macrophages are capable of killing infected or self-target cells and can also induce further downstream immune responses through the presentation of surface antigens to signal and recruit other cells and cell types (56).

A common feature of salmonellosis is the notable inflammatory response elicited by the host innate immune system. Both the host and pathogen have evolved defense mechanisms that result in a complex cross-talk that culminates with the induction of the host immune response.

Salmonella species can cross the epithelial barrier by passive transport facilitated by dendritic cells, which extend pseudopods between local epithelial cells, or by active invasion. Upon reaching the lower intestine, the bacteria will adhere to the mucosal membranes and invade epithelial cells (57). One such site where this occurs is the microfold (M) cells of Peyer's patches that are located in the small intestine where the bacteria will translocate across the epithelial barrier to the underlying follicles and mesenteric lymph nodes of the lymphoid tissue (58) (**Figures 3A,B**). During sustained bacteremia, secondary infections can occur due to the dissemination of the bacteria to other organs such as the gall bladder, liver, and spleen. The gall bladder serves as a reservoir in chronic cases of *S.* Typhi and *S.* Typhimurium infection (59, 60). Infection by invading bacteria can originate from both the blood and/or retrograde bile. Biofilm formation on gallstones is a reported avenue through which chronic carriage and shedding of *Salmonella* species can be established. These events set in motion a cycle of infection wherein bacteria basolaterally reinvade epithelial cells of the intestinal wall or are shed in feces. In time, the symptoms of salmonellosis will resolve. However, asymptomatic carriage of the bacteria can occur in patients for months or years with the potential to relapse in the future.

TRANSMISSION OF INFECTION

Following the ingestion of contaminated food, these bacteria will colonize the intestines by invading dendritic cells and enterocytes of the intestinal epithelium barrier. *Salmonella* species, which are successful in passing this barrier are confronted by proximal macrophages and may be phagocytosed, or actively invade the macrophages, using T3SS-1 and fimbriae, among other bacterial surface adhesins [H. (15)] (**Figure 3Ci**).

After being internalized by macrophages, *Salmonella* then reside within a membrane bound compartment distinct from the phagosome and lysosome known as the SCV. In this cellular compartment, *Salmonella* can survive and replicate in the absence of host antimicrobial defense mechanisms, thereby evading endosomal fusion with the NADPH oxidase complex (61) (**Figure 3Cii**).

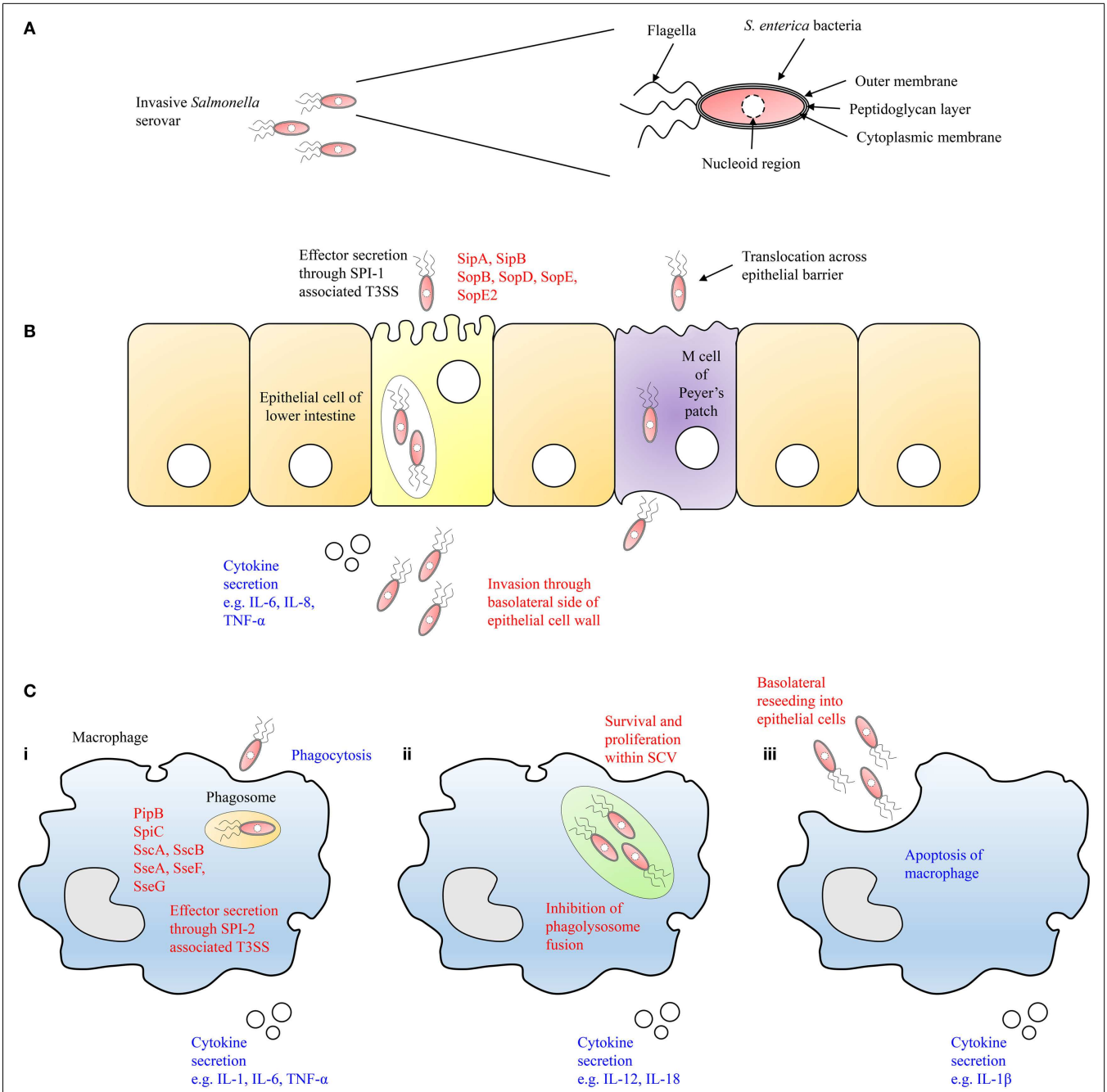

FIGURE 3 | Schematic illustration of the infection of epithelial cells of the lower intestine and macrophages by *Salmonella* is shown. **(A)** The complex membrane structure of *Salmonella* allows it to survive until reaching the epithelial cell wall of the host in the lower intestine. **(B)** *Salmonella* then translocate across M cells of Peyer's patches or actively invade epithelial cells by the secretion of effector proteins through the SPI-1 encoded T3SS-1. **(C) (i)** After crossing the epithelial barrier, *Salmonella* are engulfed by proximal macrophages that will secrete effector proteins into the cytosol of the cell via the SPI-2 encoded T3SS-2 and prevent fusion of the phagosome with the lysosome. **(ii)** Within the SCV, *Salmonella* will proliferate resulting in cytokine secretion by the macrophage. **(iii)** Finally, the macrophage will undergo apoptosis, and *Salmonella* will escape the cell to basolaterally reinvade epithelial cells or other phagocytic cells of the host innate immune system.

From within the SCVs, SPI-2 genes are expressed encoding T3SS-2, which enables *Salmonella* to translocate a range of effector proteins into the cytoplasm of the host cell including SigD/SopB, SipA, SipC, SodC-1, SopE2, and SptP leading to the rearrangement of the actin cytoskeleton. T3SS-2 has been described as necessary for systemic virulence in murine models and survival within macrophages (62). In contrast, systemic translocation of *S.* Dublin in cattle requires T3SS-1 but not T3SS-2 (63).

CYTOKINE RESPONSES AND SIGNALING

Proinflammatory cytokines including the interleukins (IL-1β and IL-6), interferons (IFN-γ), and tumor necrosis factor (TNF-α) are synthesized and these act to promote systemic inflammation (64–67). IFN-γ, also known as macrophage activating factor (MAF), plays an important role in persistent infection as it influences the duration of macrophage activation. Secretion of IFN-γ is dependent on IL-18, also known as interferon gamma inducing factor, and is essential for establishing an early host resistance to infection with *Salmonella* (65, 68).

Macrophages are involved in both the innate and adaptive immune responses. Following exposure to specific cytokines, they undergo either classical (Th1) or alternative (Th2) activation. Classical activation by bacterial LPS or IFN-γ leads to alteration in the secretory profile of the cells through production of organic nitrate compounds such as nitric oxide (NO). Alternative activation by IL-4, IL-10, or IL-13 leads to the production of polyamines and proline inducing proliferation and collagen production, respectively. The presence of *Salmonella* within these cells leads to cytokine secretion and an inflammatory reaction or programed cell death through apoptosis (69, 70) (**Figure 3Ciii**).

Cytokine signaling, induced by the interaction of the host cells and bacteria, is crucial to the development and progression of salmonellosis. Cytokines are responsible for regulating both the innate and adaptive host immune responses. The equilibrium between pro- and anti-inflammatory cytokines controls the infection preventing damage to the host from prolonged inflammation. *In vitro* cell culture of bone marrow derived macrophages and primary cell lines have shown that *Salmonella* promotes chemokine and cytokine synthesis in both dendritic and epithelial cells as well as macrophages (69, 71, 72). Cytokines have a broad range of effects upon the host cell during infection. Chemokine C–C motif ligand (CCL2), IFN-γ, IL-12, IL-18, TNF-α, and transforming growth factor (TGF-β) confer protection during infection (73). Conversely, IL-4 and IL-10 interfere with the host defense mechanisms (74).

ENVIRONMENT ADAPTATION

Salmonella adapt to the intracellular environment of phagocytic cells during infection. The transition from extracellular to intravacuolar environments involves global modulation of bacterial gene expression. The complete transcriptional landscape of intracellular *S.* Typhimurium following macrophage infection has been previously reported (75, 76). During replication in murine J774 macrophages, 919 of 4,451 *S.* Typhimurium genes are differentially expressed. Many of the *in vivo*-regulated genes are of unknown function suggesting novel macrophage-associated functions for intracellular growth (77).

It has been shown previously that *S.* Typhimurium requires glycolysis for infection of mice and macrophages and that glucose transport is required for replication within macrophages. During systemic infection of mice, *S.* Typhimurium replicates in macrophages within the SCV. Mutation of the *pfkAB*-encoded phosphofructokinase, the rate-limiting step in glycolysis, severely attenuates replication and survival within RAW 264.7 macrophages. Mutants with perturbed phosphoenolpyruvate:carbohydrate phosphotransferase systems or those unable to catabolize glucose exhibit reduced replication within RAW 264.7 macrophages (78).

Salmonella upregulates RpoS-dependent stress responses as well as other response mechanisms when challenged to grow in sublethal concentrations of the bile salt sodium deoxycholate (DOC). The latter is known to disrupt membranes, denature proteins, and damage DNA (79). It has been previously shown that *Salmonella* can pre-adapt to several stresses in order to survive the adverse conditions encountered, such as those encountered in a contaminated food matrix and any associated food production processes. Similarly, the subsequent ingestion of the bacterium by the host presents an array of challenges to the organism including acid, cold, osmotic, and peroxide stress (80).

PATHOLOGICAL SYMPTOMS

Prolonged activation of the innate immune system can have adverse effects, which include intravascular coagulation, systemic inflammation, and tissue injury. In severe cases, these symptoms can lead to death. An aggressive proinflammatory response to infection with *Salmonella* is not a common occurrence and it arises rarely in patients with typhoid fever. Unusual cases leading to intravascular coagulation do not present with readily recognizable clinical signs (81, 82). In these cases, the blood serum levels of IL-1β and TNF-α are lower when compared to that of patients infected with other Gram-negative bacteria (83).

Individuals suffering from typhoid fever exhibit a distinct peripheral blood metabolite profile, which has been elucidated by both microarray and transcriptional profiling techniques (66, 84). This profile diminishes following treatment and upon recovery the majority of individuals exhibit a peripheral blood profile similar to that of uninfected controls. Those who do not develop a typical peripheral blood profile following treatment may possess genetic mutations that render them incapable of mounting an appropriate immune response. These patients have been shown to be prone to relapse, reinfection, and in some cases become carriers (66).

IMMUNODEFICIENCY

There has been no evidence to support a correlation with susceptibility to typhoid fever and primary or acquired immunodeficiency. This is in contrast to infection with NTS serovars where infection causes high levels of morbidity and mortality in patients with primary or acquired immunodeficiencies such as HIV infection. It has been proposed that this difference is attributed to the manner in which signaling occurs via the PRRs. The production of IL-17 by T-helper 17 cells (Th17) among other cytokines (IL-21, IL-22, and IL-26) is important for the dissemination of NTS serovars but not *S.* Typhi (85, 86).

MODELS OF INFECTION

S. Typhi is a host-adapted pathogen, which infects humans causing typhoid fever. Investigating the interactions of this pathogen with the host has proved challenging as there are few animal models for typhoid fever that are of direct relevance to their human infection counterpart. This problem has been partially alleviated by the establishment of the murine *S.* Typhimurium infection model, which has been used to study typhoid fever. The immune responses and subsequent inflammation mounted

by mice following an *S.* Typhimurium infection mimics those observed in human patients with typhoid fever as well as the subsequent intestinal pathology (87). Mice are inoculated orally or systemically by intravenous or intraperitoneal injection in addition to optional streptomycin pre-treatment (88). *S.* Typhimurium induced colitis in streptomycin-pre-treated mice is reminiscent of many symptoms of the human infection counterpart including epithelial ulceration and infiltration of PMN/CD18(+) cells (89). A comparison between streptomycin-pre-treated and untreated mice highlighted the drastic influence of streptomycin on resistance to colonization by *S.* Typhimurium whereby 100% of treated and none of the untreated mice excreted the bacterium in their feces (90). A disease with features reminiscent of typhoid fever can be observed in BALB/c or C_{57}BL/6 mice when inoculated with *S.* Typhimurium due to a mutation in the *SLC11A1* gene, which encodes natural resistance-associated macrophage protein one (Nramp1). In contrast to this, chronic and persistent carrier states of infection can be studied using Nramp+/+ mice as they are resistant to infection with *S.* Typhimurium (25, 88). However, there has been no correlation identified in humans between *Nramp* alleles and susceptibility to typhoid fever as *S.* Typhimurium causes less severe disease symptoms in humans to that of *S.* Typhi. As a result, conclusions drawn from animal experiments must be interpreted carefully (91). Furthermore, it has been reported that *tlr11*$^{-/-}$ mice are more susceptible to infection by *S.* Typhimurium and can be infected with *S.* Typhi, which typically does not cause infection as TRL11 is normally expressed in mice but not in humans (92). Recently, an alternative *S.* Typhi murine model, which resembles human typhoid fever, was established using non-obese diabetic (NOD)-SCID IL2rγ^{null} mice, which have been humanized by engrafting human hematopoietic stem cells (hu–SRC–SCID mice). This model results in lethal infection with inflammatory and pathological responses, which mimic human typhoid fever (93).

As well as murine models of infection, the larvae of the wax moth *Galleria mellonella* (*G. mellonella*) have been used to study host–pathogen interactions with *Salmonella* species. Isogenic mutant strains of *S.* Typhimurium lacking known virulence determinants were tested to identify their role in pathogenicity. Interestingly, mutants depleted of either or both SPI encoded T3SS-1 and T3SS-2 exhibited no alterations in their virulence phenotype. Attenuation of the PhoPQ two-component signal transduction system resulted in reduced pathogenicity due to the lack of phoQ (94). As reported in murine models, mutations in the *hfq* gene, which encodes the chaperone protein Hfq that plays an important role in the binding of regulatory sRNA transcripts to their antisense targets attenuated the pathogenicity of *S.* Typhimurium in *G. mellonella.* Endoribonuclease RNase E and RNase III mutants show an attenuated virulence phenotype including impairment in motility and reduced proliferation inside *G. mellonella* (95).

Recently, zebrafish (*Danio rerio*) models have provided a unique opportunity to study the function of phagocytic cells such as neutrophils and macrophages. Transgenic zebrafish lines with fluorescently labeled leukocyte populations enable non-invasive imaging of the mechanisms by which different pathogens interact with macrophages and evade the host innate immunity (96). Similarly, 28 h old zebrafish embryos infected with DsRed labeled *S.* Typhimurium allowed for the precise location of the pathogen to be determined in a living host over a 3 day time course using multidimensional digital imaging microscopy. Lethal infection with *S.* Typhimurium residing and proliferating in both the endothelium layer of blood vessels and macrophages was observed (97).

FUTURE PERSPECTIVES

To date, there have been many studies elucidating the complex *Salmonella*–host interactome. Our understanding of the virulence determinants of *Salmonella* species and their mechanisms of action has been extended by the utilization of murine, *G. mellonella*, and zebrafish models of *S.* Typhimurium infection in addition to *ex vivo* cell culture methods. Despite this, further work is needed to determine the specific contribution of many of these regulators and virulence factors for which clear functions and roles have yet to be defined. Characterizing the pathogenesis of salmonellosis will be crucial to the development and implementation of future therapeutic strategies to treat this illness. The importance of which has been recently highlighted in reports on the emergence of antimicrobial resistance in *Salmonella* and many other bacterial pathogens (98).

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Mucosal inflammatory response to *Salmonella typhimurium* infection

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The human intestinal epithelium consists of a single layer of epithelial cells that forms a barrier against food antigens and the resident microbiota within the lumen. This delicately balanced organ functions in a highly sophisticated manner to uphold the fidelity of the intestinal epithelium and to eliminate pathogenic microorganisms. On the luminal side, this barrier is fortified by a thick mucus layer, and on the serosal side exists the lamina propria containing a resident population of immune cells. Pathogens that are able to breach this barrier disrupt the healthy epithelial lining by interfering with the regulatory mechanisms that govern the normal balance of intestinal architecture and function. This disruption results in a coordinated innate immune response deployed to eliminate the intruder that includes the release of antimicrobial peptides, activation of pattern-recognition receptors, and recruitment of a variety of immune cells. In the case of *Salmonella enterica* serovar typhimurium (*S. typhimurium*) infection, induction of an inflammatory response has been linked to its virulence mechanism, the type III secretion system (T3SS). The T3SS secretes protein effectors that exploit the host's cell biology to facilitate bacterial entry and intracellular survival, and to modulate the host immune response. As the role of the intestinal epithelium in initiating an immune response has been increasingly realized, this review will highlight recent research that details progress made in understanding mechanisms underlying the mucosal inflammatory response to *Salmonella* infection, and how such inflammatory responses impact pathogenic fitness of this organism.

Keywords: *Salmonella typhimurium*, mucins, microbiota, epithelial barrier, immune recognition, neutrophil recruitment, mucosal inflammation, PMN transmigration

INTRODUCTION

Salmonella enterica serovar typhimurium (*S. typhimurium*) is a Gram-negative, facultative, intracellular anaerobe that causes severe inflammation of the intestinal mucosal epithelium resulting in gastroenteritis. *S. typhimurium* causes disease through its primary virulence mechanism, the type III secretion system (T3SS). There are two T3SSs that are encoded by two regions of the bacterial chromosome called *Salmonella* pathogenicity island 1 and *Salmonella* pathogenicity island 2 (SPI-1 and SPI-2). These pathogenicity islands also encode effector proteins that are secreted from the T3SS and translocated into epithelial cells at the mucosal surface of the intestine. Upon contact with the mucosal epithelium, SPI-1 encoded effector proteins are translocated into epithelial cells and promote bacterial entry and inflammation. SPI-2 encoded effector proteins generally function to maintain the intracellular survival of *S. typhimurium* after the organism has been macropinocytosed by epithelial cells. More recent studies, however, suggest that SPI-1 and SPI-2 effector proteins may not be as functionally compartmentalized as originally thought (1–3).

The architecture of the mucosal epithelium contains several barriers that attempt to prevent or impede infection by pathogenic bacteria. Mechanisms of protection are employed by all of these barriers in order to maintain the integrity of the epithelial cell monolayer and limit inflammation-associated damage (**Figure 1**). *S. typhimurium* can modulate the signaling pathways that govern these mechanisms, including targeting specific proteins or inducing pathways through functional mimicry, in order to provide itself with an ecological advantage with its T3SS virulence mechanism. Although *S. typhimurium* can, in certain instances, bypass the innate immune response, the adaptive inflammatory immune response is in most instances capable of clearing the pathogen, albeit with increased damage to the mucosal epithelium.

ARCHITECTURE OF THE MUCOSAL EPITHELIUM: BARRIERS AGAINST INFECTION

MUCUS/MUCINS

The luminal side of the intestinal epithelium is covered with a thick layer of mucus primarily composed of mucins, the main secretory product of goblet cells (**Figure 1**). Mucins are high molecular weight glycoproteins that aggregate to form a "gel-like" barrier to defend against endogenous or exogenous luminal insults. To date, at least 17 highly conserved mucins have been identified, each with varying specificities for different epithelial tissues [for review, see Ref. (4)]. Furthermore, these mucins have been categorized into two major groups: cell surface mucins and secreted mucins (**Figure 1**). Of these two categories, it is the secreted mucins that form the major structural component of the mucosal layer, and out of the known secreted mucin proteins, MUC2, MUC5AC, MUC5B, MUC6, and MUC19 are classified as gel-forming for

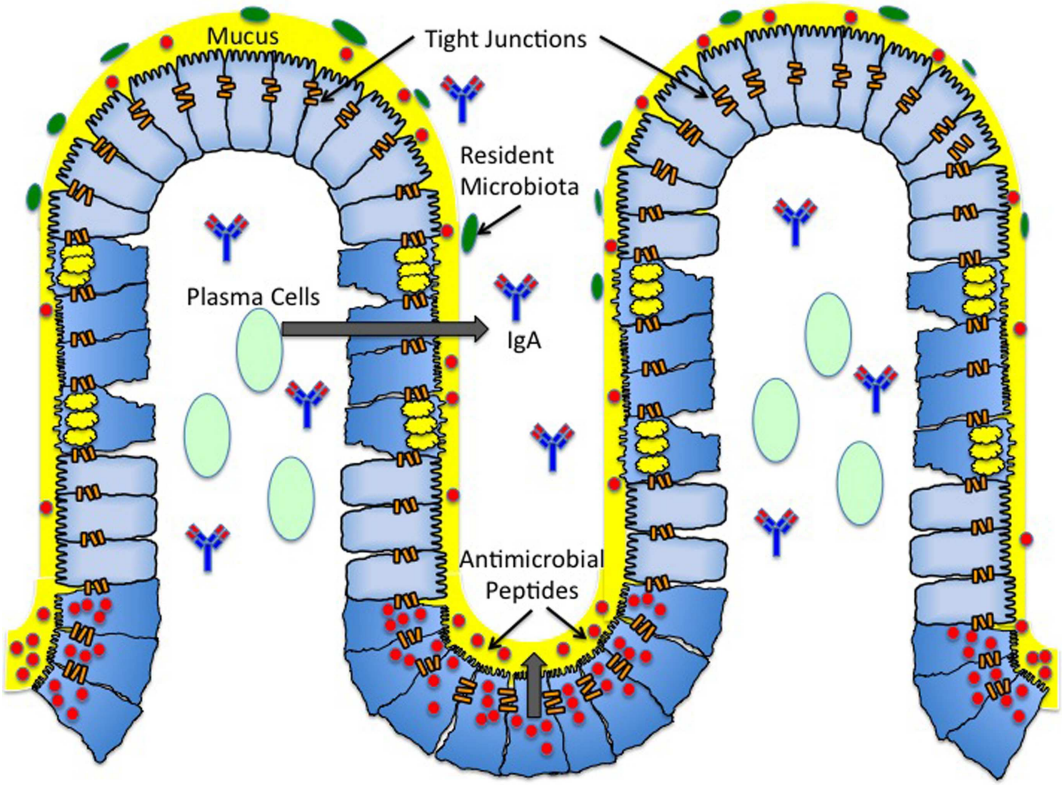

FIGURE 1 | Architecture of the mucosal surface. The mucosal surface of the intestine contains a single layer of epithelial cells. The monolayer of epithelial cells is fortified by a layer of mucus (yellow) produced by *Goblet cells* (*blue cells with yellow granules*). This thick mucus layer contains membrane bound and secreted mucins. The antimicrobial peptides (red) secreted by *Paneth cells* (*blue cells with red granules*) reside in the thick mucus layer, providing another form of protection against both pathogenic and commensal bacteria. Antimicrobial peptides include defensins, cathelicidins, and histatins. *Plasma B cells* (*light green*) reside in the subepithelial region and produce *secretory IgA* (*blue and red antibody*). Secreted IgA is found in the subepithelial region and the lumen. *Resident microbiota* (*green*) reside in the outer mucus layer, providing yet another barrier to pathogenic infection. The majority of resident microbiota belong to two phyla – *Firmicutes* and *Cytophaga–Flavobacterium–Bacteroidetes*. The seal between epithelial cells is maintained by *tight junctions* (*orange bars*). Tight junctions are dynamic structures composed of zonula occludens and junctional adhesion molecules.

human mucosal surfaces [for review, see Ref. (5, 6)]. The predominant mucin comprising the mucus layer of the intestinal epithelium is MUC2, although MUC5AC has been shown to be expressed in the mucus layer of the fetal intestine (7).

The mucosal layer consists of an inner layer of mucus that is firmly adherent to the intestinal epithelial cells (mainly comprised of cell surface mucins) and a looser outer layer of mucus (mainly comprised of secreted mucins) [for review, see Ref. (8)]. For quite some time, the mucus layer of epithelial surfaces was thought to solely serve the purpose of providing a physical barrier, preventing access of pathogenic bacteria or resident microbiota to the epithelial cells. However, it has been increasingly realized that the mucins in the outer sublayer of the mucosal barrier also provide an energy source for both resident microbiota and pathogenic microorganisms capable of adhering to the mucus layer. This layer provides both commensal and pathogenic microorganisms with a niche in which to grow and colonize the intestine [Ref. (9); for review, see Ref. (10)]. The inner layer of the mucosal surface is considered "sterile," largely due to the presence of antimicrobial peptides secreted by Paneth cells (**Figure 1**, discussed later), thereby limiting bacterial colonization to the outer mucus layer [Ref. (8); for review, see Ref. (11, 12)].

Certain cell surface mucins in the inner mucus layer also directly play a role in protecting against bacterial colonization on the epithelial surface by acting as pathogen-binding decoys. For example, epithelial cells can release Muc1 (called mucin shedding) in response to *Helicobacter pylori* infection, and Muc1 will bind the bacteria, preventing its adhesion to the intestinal epithelium (8). Furthermore, it has been shown that approximately fivefold more *H. pylori* colonize the intestinal epithelium of *Muc1*$^{-/-}$ mice than wild-type mice (13). Although the thick mucus layer provides protection in the form of a physical barrier, it is significant to note that the necessity to maintain healthy intestinal microflora does provide pathogenic bacteria with the same energy source and corresponding growth advantage as well. This advantage has allowed certain pathogenic bacteria to develop mechanisms to circumvent the protection provided by the mucus sublayers and infect the underlying epithelial cells. As an example, certain pathogenic *Escherichia coli* (*E. coli*) secrete mucinolytic proteins, thus allowing them to persist and colonize within the mucus

layer (14, 15). Unlike *E. coli*, *S. typhimurium* does not enzymatically degrade mucus in order to colonize the mucosal epithelium. Rather, mucins have actually been shown to be the binding sites for *S. typhimurium*, and in particular a 250-kDa neutral mucin has been implicated as a receptor for *S. typhimurium* (9).

RESIDENT MICROBIOTA

The mammalian intestinal microflora contains ~10^{14} resident bacteria, comprising ~1,000 species, and they reside in the outer sublayer of the mucosal barrier on the luminal side of the intestinal epithelium (**Figure 1**). The vast majority (~90%) of the commensal bacteria in humans and mice belong to two phyla: *Firmicutes* and *Cytophaga–Flavobacterium–Bacteroidetes*. Though much of the resident microbiota are of the same two phyla, there are differences in intestinal floral composition of individuals that arise at the species level (16). Diversity of the intestinal microflora is susceptible to change due to environmental factors such as nutrition, and there is variation (increases/decreases in quantity of certain species of bacteria or increases/decreases in diversity of a particular genus of bacteria) in microbiota populations within different age groups (17, 18).

The resident microbiota promote resistance to infection by pathogenic microorganisms in several ways. First, they serve as a microbial barrier by competing with pathogens for resources at the outer mucosal sublayer, thereby limiting pathogenic bacterial colonization (8). Additionally, end products of metabolic pathways of individual species of bacteria have been shown to prevent pathogenic infection. For example, *Bifidobacteria* carbohydrate metabolism produces high concentrations of acetate, which has been shown to prevent release of Shiga toxin during infection with enterohemorrhagic (EHEC), thereby decreasing the risk of toxin gaining access to the blood stream from where it can otherwise cause lethal damage to target organs such as the kidneys (19). Along the same lines, it is becoming more appreciated that the composition of the intestinal microbiota may be just as important in defending against infection as the quantity of the commensal bacteria. For instance, selective reduction of *Lactobacilli* and *Enterococci*/group D streptococci groups of bacteria through the use of low concentrations of antibiotics has been shown to make mice more susceptible to colonization of the epithelial surface with *S. typhimurium* without drastically affecting the overall numbers of commensal bacteria (16). Further investigation is needed to determine exactly which resident microbiota are necessary to prevent other pathogens from colonizing the intestinal epithelium, especially since certain enteric pathogens have developed mechanisms to subvert this microbial form of protection.

Nevertheless, an emerging concept is that inflammation of the mucosal epithelium plays a role in the bacterial fitness of *S. typhimurium*. One of the more basic advantages of *S. typhimurium*-induced inflammation is that the clinical manifestation of diarrhea facilitates the spread of bacteria. Additionally, it has been shown that unlike avirulent strains, wild-type *S. typhimurium* is capable of out-competing commensal microbiota in re-colonization experiments after treatment with antibiotics. Furthermore, *S. typhimurium* exploits inflammation to promote its own colonization. In this instance, *S. typhimurium* has been shown to out-compete the resident microbiota in a mouse colitis model (20). One explanation for this phenomenon is that inflammation provides *S. typhimurium* with a respiratory electron acceptor that members of the resident microbiota are unable to utilize. In particular, reactive oxygen species generated by neutrophils (PMNs) during inflammation can react with endogenous thiosulfate to form tetrathionate, a respiratory electron acceptor (21). The ability to respire tetrathionate has been mapped to the *ttrRSBCA* locus, which is located in SPI-2 (22). Under anaerobic conditions in which thiosulphate was oxidized to tetrathionate, *S. typhimurium* displays a growth advantage in comparison to resident microbiota under the same conditions (21).

Both resident microbiota and *S. typhimurium* compete for resources available for fermentation at the mucosal layer; however, resident microbiota are incapable of using the fermentation end products (21). By reducing the tetrathionate made available by the inflammatory response to infection, *S. typhimurium* is capable of respiring the fermentation end products in anaerobic conditions, thereby providing it with an advantage over the resident microbiota (21). Remarkably, the growth benefit is conferred to *S. typhimurium* only in the presence of inflammation, and it has been suggested that a reason *S. typhimurium* has evolutionarily maintained its inflammation-inducing virulence mechanisms could be to provide it with an ecological advantage at the mucosal surface of the intestine (21).

PANETH CELLS

Paneth cells are specialized epithelial cells located at the base of crypts of Lieberkuhn that generate and secrete antimicrobial peptides of ~20–40 amino acids in length (**Figure 1**). There are four families of antimicrobial peptides: defensins, cathelicidins, histatins, and lactoferrin (**Figure 1**, **Table 1**). Defensins are positively charged and directly interact with the negatively charged membrane of pathogenic microorganisms resulting in membrane destabilization and pore formation. Cathelicidins are also positively charged, and they function in binding and neutralizing lipopolysaccharides (LPS), ultimately resulting in pore formation. Unlike defensins and cathelicidins, histatins do not interact with the membranes of pathogenic bacteria. Instead, histatins are ingested by the bacteria, inhibit mitochondrial respiration, and kill the microorganism by generating reactive oxygen species [for review, see Ref. (23)]. Lactoferrin is a cationic protein that sequesters iron, an essential nutrient for pathogenic

Table 1 | Summary of antimicrobial peptides.

Antimicrobial peptides	Function	Reference
Defensins (i.e., HD-5, HD-6)	Destabilization of bacterial membranes	(23, 25, 26)
Cathelicidins (i.e., CRAMP, LL-37)	Neutralization of LPS	(23, 27, 28)
Histatins	Generation of reactive oxygen species	(23)
Lactoferrins	Sequestration of iron and destabilization of bacterial membranes	(23, 24)

microorganisms. Additionally, lactoferrin can bind LPS and destabilize bacterial membranes similar to defensins and cathelicidins (23, 24). The antimicrobial activities of these peptides are nonspecific, as their activity provides a first line of defense against both Gram-positive and Gram-negative bacteria, fungi, and enveloped viruses.

All antimicrobial peptides are produced in an inactive, prepropeptide form and must be processed (i.e., enzymatically) either intracellularly or extracellularly to become active (10). For example, the alpha-defensin HD-5 is stored in Paneth cells in an inactive, pre-propeptide form and is processed by trypsin into its active form (29). Antimicrobial peptide production has been shown to be upregulated in response to bacteria (30). However, pathogenic microorganisms have developed methods to counteract the effectiveness of the antimicrobial peptides. Examples of these methods include covalently modifying the bacterial cell membrane to reduce its net negative charge, using bacterial proteases to catalytically inactivate the antimicrobial peptides, and using ATP-driven pumps to physically remove the antimicrobial peptides from the bacterial cytoplasm [for review, see Ref. (31)]. Certain pathogens have developed resistance to the antimicrobial activities of the peptides secreted by Paneth cells in order to promote their intracellular survival.

Antimicrobial peptides that provide protection against *S. typhimurium* infection have been identified using transgenic mouse models. Alpha-defensin HD-5 transgenic mice were shown to consistently have a significant reduction in the *S. typhimurium* burden in the distal intestine and spleen in comparison to wild-type mice that do not express this antimicrobial peptide, indicating the antimicrobial activity of HD-5 conferred the transgenic mice with an enhanced ability to kill *S. typhimurium* in the intestinal lumen (26). Another alpha-defensin shown to provide increased defense against *S. typhimurium* infection is HD-6, which binds bacterial membrane proteins, thereby inhibiting contact of *S. typhimurium* with epithelial cells (25). Since HD-6 does not kill *S. typhimurium*, HD-6 transgenic mice do not display the decrease in bacterial burden seen with HD-5 transgenic mice; however, HD-6 transgenic mice display a profound increase in survival rate in comparison to wild-type mice that do not express this antimicrobial peptide, indicating the antimicrobial activity of HD-6 must act in concert with another defense mechanisms at the mucosal barrier to eliminate *S. typhimurium* (25).

In addition to defensins, mouse models have also identified the significance of cathelicidins and lactoferrin. The sole murine cathelicidin called cathelin-related antimicrobial peptide (CRAMP) has been shown to impair intracellular replication of *S. typhimurium in vivo* and *in vitro* (27). Additionally, *S. typhimurium* displayed enhanced survival in macrophages derived from CRAMP-deficient mice (27). CRAMP is similar in structure and antimicrobial properties to the only human cathelicidin called LL-37, which has been shown to display a broad spectrum of activity against bacteria including *S. typhimurium* (28). A recent study identified the *in vivo* effect of lactoferrin on *S. typhimurium*. In this study, mice treated with bovine lactoferrin displayed a reduction in severity, mortality, and inflammation during infection, indicating the antimicrobial properties of lactoferrin are significant for defense against *S. typhimurium* (24).

THE EPITHELIAL BARRIER

In addition to mucosal defenses described above, interactions between cells of the epithelial cells in the monolayer also provide a barrier against bacterial entry. Tight junctions are dynamic structures composed of zonula occludens (ZO) and junctional adhesion molecules that effectively adhere the cells of the epithelial monolayer to each other (**Figure 1**) (8). The integrity of this seal is maintained by the interaction of tight junction components with the actin cytoskeleton. However, the permeability of this seal is regulated by physiological conditions, and it therefore can be manipulated. For example, treating epithelial monolayers with inflammatory cytokines, such as IL-1β, increases the permeability of the tight junctions (32). The increase in tight junction permeability can facilitate the translocation of bacteria from the lumen to the subepithelial region, making them a target for pathogenic manipulation. Pathogenic microorganisms can accomplish the manipulation of tight junctions by usurping signaling pathways, such as the Rho-GTPase pathway, which regulates actin cytoskeleton rearrangement (8).

Salmonella typhimurium infection has been shown to regulate certain tight junction proteins, which ultimately promotes translocation of the bacteria through the epithelial cell monolayer (33). Upon infection with *S. typhimurium*, occludin becomes dephosphorylated and subsequently removed from epithelial tight junctions (33). Additionally, ZO-2 is recruited from the cytosol to membrane, indicating *S. typhimurium* alters the intracellular distribution of this tight junction protein (33). Surprisingly, ZO-1, which is normally regulated by pathogens in a similar manner to ZO-2, appears to be degraded during *S. typhimurium* infection (33). Manipulation of tight junction proteins serves to disrupt the epithelial barrier by increasing its permeability, thereby allowing *S. typhimurium* to more effectively invade the basolateral side of the epithelial cell monolayer.

In order to mount a successful infection, *S. typhimurium* must disrupt some aspects of the protective mechanisms employed by the mucosal epithelium. As mentioned previously, the two T3SS and the secreted bacterial effector proteins promote entry, inflammation, and intracellular survival. In addition, in order to subvert the action of antimicrobial peptides, *S. typhimurium* uses the two-component system PhoQ/PhoP, which regulates the expression of SPI-2 encoded genes as well. Specifically, PhoP/PhoQ regulators promote remodeling of the bacterial envelope, resulting in increased resistance to antimicrobial peptides that recognize LPS. Furthermore, the PhoP/PhoQ regulators repress transcription of genes for the T3SS, in attempt to avoid detection, and induce protective mechanisms against hydrogen peroxide (10). In addition to rearranging the actin cytoskeleton and targeting specific tight junction proteins, *S. typhimurium* also manipulates tight junctions via the action of SipA, SopE, SopE2, and SopB (8). These effector proteins induce Rho-GTPase activation, and inhibition of this effector-induced Rho-GTPase activation prevents tight junction disruption (8).

The manipulation of tight junctions has also recently been shown to facilitate the transmigration of PMNs across the epithelial cell monolayer (33). The primary mechanism of PMN migration in *S. typhimurium* infection involves the recruitment of neutrophils into the subepithelium and the formation of a

chemoattractant gradient that directs the neutrophils into the lumen (**Figure 2**, discussed in detail later). However, recent research implicates the disruption of tight junctions in facilitating PMN migration even in the absence of the chemoattractant gradient (33). Thus, *S. typhimurium* not only modulates the release of neutrophil chemoattractants that induce PMN migration, but also directly influences the tight junctions that maintain the fidelity of the epithelial cell monolayer in order to promote bacterial translocation and PMN transepithelial migration.

LAMINA PROPRIA

The lamina propria is the connective tissue underlying the epithelial cell monolayer. It contains multiple immune effector cells including B cells, T-cells, dendritic cells, natural killer (NK) cells, macrophages, eosinophils, and mast cells. If enteric pathogens are capable of surmounting the barriers described above and penetrate the intestinal epithelium, a coordinated immune response utilizing these immune effector cells is activated. Sampling of luminal antigens occurs in specialized cells called M cells, which transport the antigens to a subepithelial region where the antigen comes in contact with dendritic cells. Dendritic cells bound to antigen then migrate to the mesenteric lymph node to present the luminal antigens to naïve T-cells and B cells. These naïve lymphocytes then differentiate into several effector cells including CD8 cytotoxic T-cells, CD4 helper T-cells, regulatory T-cells, and antibody secreting B cells. Although this marks the beginning of a coordinated immune response to pathogenic bacteria, the resting lamina propria does have protective functions that provide an added layer of defense prior to the full activation of the mucosal immune system.

The most abundant B cell found in the lamina propria is the IgA-secreting B cell (**Figure 1**). Secreted IgA is also the primary secreted immunoglobulin found in the thick mucus layer. One of the main roles of secreted IgA is a process called immune exclusion, which includes prevention of pathogens from adhering to the mucosal surface on the luminal side of the intestinal epithelium and removal of antigens from the basolateral side of the intestinal epithelium. On the luminal side, secreted IgA primarily interferes

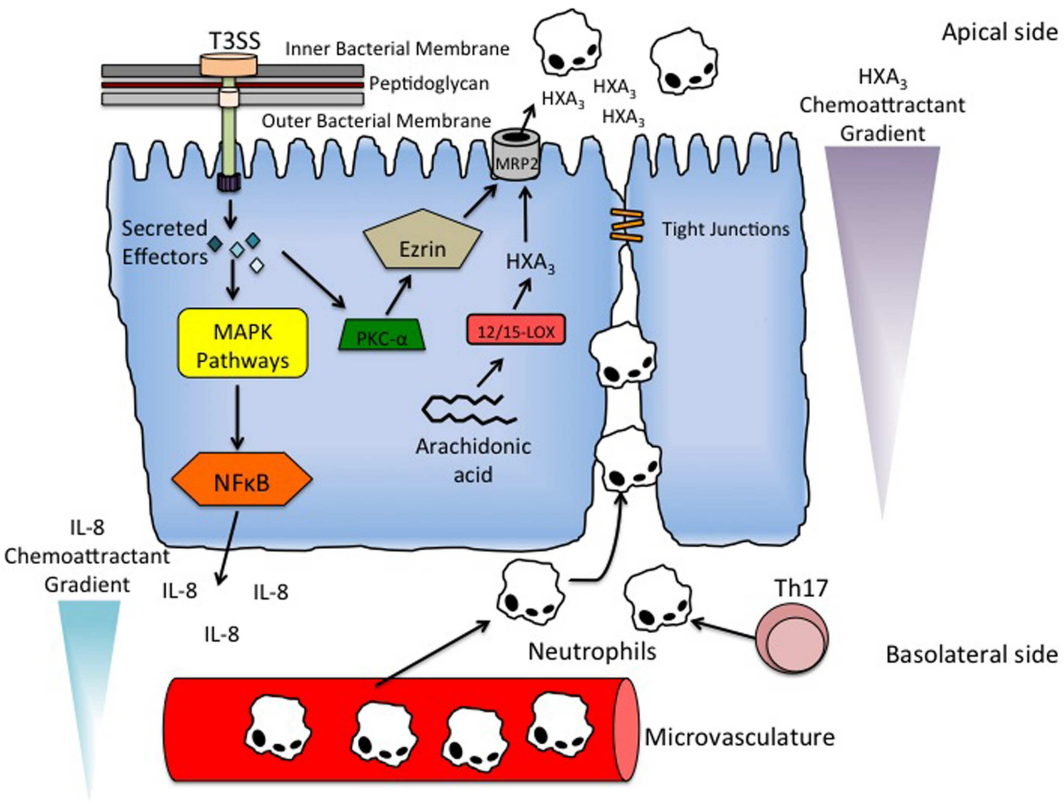

FIGURE 2 | Mechanism of PMN recruitment and PMN transmigration. *S. typhimurium* utilizes its T3SS to secrete effector proteins into epithelial cells to activate inflammatory signaling pathways. In particular, the activation of Rho-GTPases by SopE, SopE2, and SopB result in the induction of mitogen-activated protein kinase (MAPK) pathways. The stimulated pathways include ERK, JNK, and p38, resulting in the terminal activation of major inflammatory regulator NF-κB. Activation of NF-κB results in the basolateral secretion of IL-8 producing a chemoattractant gradient that recruits neutrophils to the subepithelial region from the underlying microvasculature. Th17 cells are also present in the subepithelial region, and function to recruit and activate neutrophils in the subepithelium. PMN transmigration is facilitated by another chemattractant HXA_3. HXA_3 is a bioactive eicosanoid that is synthesized from arachidonic acid via the 12/15-lipoxygenase pathway in epithelial cells. It is secreted into the lumen via the action of an ATP-binding cassette transporter called MRP2. *S. typhimurium* effector protein SipA stimulates the recruitment of PKC-α to the apical membrane, which in addition to the ERM protein ezrin, modulate the localization of MRP2 to the apical membrane, thereby allowing secretion of HXA_3 into the lumen and production of the chemoattractant gradient that induces PMN transmigration.

with microbial adhesins, whereas on the basolateral side secreted IgA functions in an export mechanism by binding antigens and shuttling them back across the epithelial monolayer into the lumen (34). Secreted IgA does not activate an inflammatory immune response when it neutralizes pathogenic microorganisms, thereby upholding the integrity while preventing inflammation-induced damage of the mucosal epithelium (35).

Secreted IgA also has a direct effect on the virulence mechanisms of certain pathogens. In *S. typhimurium* infection, it has been shown that a monoclonal, polymeric IgA antibody Sal4 binds the O-antigen (O-Ag) component of LPS on the bacterial membrane, resulting in its destabilization [Ref. (36); for review, see Ref. (37)]. Recent evidence indicates that the bacterial membrane destabilization results in impaired T3SS translocon formation, decrease in effector protein delivery, and decrease in flagellum-based motility (36). *S. typhimurium* responds to the binding of Sal4 to O-Ag by triggering exopolysaccharide (EPS) production and biofilm formation, though this response renders the bacteria non-invasive and avirulent (38). The mechanism of EPS production and biofilm formation has been attributed to the activation of a cyclic dimeric guanosine monophosphate-dependent pathway via an inner membrane diguanylate cyclase YeaJ (38). Furthermore, it has been suggested that the triggering of this pathway by *S. typhimurium* could be a mechanism to restore membrane stability, as EPS production could serve to shed IgA antibody or increase resistance to other luminal insults (38).

The resting lamina propria contains a heterogeneous population of CD8 cytotoxic T-cells, CD4 helper T-cells, and regulator T-cells even in the absence of pathogenic infection. The number of effector T-cells in the resting lamina propria would in any other tissue indicate an inflammatory response; however, the amount of T-cells present in the mucosal tissue of the gut is more indicative of constant immune surveillance and recognition than chronic inflammation. The cytokines produced by these T-cells maintain the mutualistic response to resident microbiota, stimulate production of IgA, induce secretion of antimicrobial peptides, and promote epithelial repair. Additionally, in the absence of pro-inflammatory cytokines (which are usually produced by innate immune cells in the presence of pathogen), the dendritic cells in the resting lamina propria contribute to maintaining tolerance to non-pathogenic antigens by promoting the production of CD4 regulatory T-cells. Regulatory T-cells produce immunosuppressive cytokines that inhibit T-cell proliferation and dendritic cell differentiation, which prevents unnecessary immune response to innocuous antigens (35).

In addition to a heterogeneous population of T-cells, the macrophages in the resting lamina propria play a key role in host defense against *S. typhimurium* infection, as well. The macrophages of the resting lamina propria can be divided into two different classes: M1 ("classically activated"), and M2 ("alternatively activated") polarized macrophages. M1 polarized macrophages are pro-inflammatory and display high phagocytic and antimicrobial activity, whereas M2 polarized macrophages are anti-inflammatory and display low phagocytic and antimicrobial activity. Hence, with this type of opposing macrophage regulation it is considered that M1 macrophages function in the clearance of infection versus M2 macrophages that assist in wound healing and suppression of T-cell function. Manipulation of macrophage polarization by *S. typhimurium* has become increasingly realized as a defense mechanism against bacterial clearance [for review, see Ref. (39, 40)]. A recent study demonstrated that the SPI-1 T3SS enables *S. typhimurium* to guide macrophage toward the M2 polarization (40). This type of control permits *S. typhimurium* to escape the more hostile environment of M1 polarized macrophages, resulting in a macrophage-specific decrease in pro-inflammatory signaling (40).

The mechanism of immunity to invasive *Salmonella* is still disputed, specifically the relevance of cell-mediated versus humoral immunity. The debate is complicated by attempts to compare different experimental models, which vary in route of *Salmonella* administration and/or susceptibility of mouse strains to *Salmonella*. In terms of cellular immunity, mice deficient for TCR α/β, MHC class II, or interferon-γ (IFN-γ) receptor fail to clear a primary *Salmonella* infection that can be resolved in normal mice (41, 42). Recently, it has also been shown that $Thy1^+$ NK cells are essential for the early production of IFN-γ during control of *Salmonella* infection (43). $CD8^+$ T-cells seem to also play a role in *Salmonella* clearance (44). It has also been documented that *Salmonella* infection promotes the expansion of intestinal intraepithelial lymphocytes (iIELs) and the activation of particularly, $CD8^+$ $TCR\gamma\delta^+$ iIELs, which in turn trigger cytolytic activity against *Salmonella*-infected epithelial cells (44).

The role of antibody-producing immune cells or B cells against *Salmonella* is still controversial (45–47). Some reports have shown the importance of antibody production and T-cell activation for protection from virulent *Salmonella* (45, 46). However, another study reported that the protective immunity provided by an attenuated *S. typhimurium* strain required B cells independently of antibody production, proposing that they confer protective immunity by presenting antigen to T-cells and acting as a source of inflammatory cytokines (47). It has also been demonstrated that transfer of immune serum into B cell-deficient mice can partially but not completely provide protective immunity (48).

THE TYPE III SECRETION SYSTEM: CO-OPTING HOST PATHWAYS TO PROMOTE ENTRY AND IMMUNE EVASION

Upon contact of *S. typhimurium* with the epithelial cell monolayer, the SPI-1 effector proteins SopE, SopE2, and SopB initiate the process of bacterial entry by activating host cell Rho-GTPases resulting in actin rearrangements (49, 50). SipA is another SPI-1 effector protein that antagonizes actin depolymerizing agents and tethers actin monomers together to form membrane ruffles, which promotes bacterial internalization (51). *S. typhimurium* is engulfed by epithelial cells through a macropinocytosis event termed bacterial-mediated endocytosis, and is ultimately contained within in a membrane-bound vesicle called a macropinosome [more commonly termed the *Salmonella* containing vacuole (SCV)]. Although prior studies thought that SopB was the sole mediator of macropinosome formation, a cooperative interaction regulated by the phosphatase activity of SopB has implicated SopD as another mediator of this process (49).

Salmonella typhimurium also targets antigen-sampling microfold (M) cells to translocate across the gut epithelium. M cells constitute a small subset of highly specialized follicle-associated

epithelium (FAE) enterocytes overlying lymphoid follicles in the gut, and are characterized by an irregular brush border, a reduced glycocalyx and lysosomal apparatus, and are programed to efficiently transcytose a wide variety of macromolecules and microorganisms from the gut lumen to the underlying immune inductive Peyer's patches (PPs) (52). Recent evidence shows the *S. typhimurium* type III effector protein SopB also induces an epithelial–mesenchymal transition of the FAE into M cells. This cellular transdifferentiation is a result of SopB-dependent activation of Wnt/β-catenin signaling leading to induction of both receptor activator of NF-κB ligand (RANKL) and its receptor RANK. The autocrine activation of RelB-expressing FAE enterocytes by RANKL/RANK induces the EMT-regulating transcription factor Slug that marks epithelial transdifferentiation into M cells. Thus, *S. typhimurium* may also transform primed epithelial cells into M cells to promote host colonization and invasion (52, 53).

Following bacterial entry of mucosal epithelia, *S. typhimurium* employs a second set of SPI-1 effector proteins to ensure repair of the actin cytoskeleton. SptP is one such effector that is directly responsible for reversing the affects of SopE and SopE2. SptP promotes restoration of the epithelial cell membrane by functioning as a GTPase-activating protein for the Rho-GTPase proteins Rac-1 and Cdc42 (54). Similarly, as several of the early SPI-1 effectors induce inflammation of the mucosal epithelium, there are effector proteins that have an anti-inflammatory function, providing *S. typhimurium* with a form of regulatory control over the inflammatory state of the mucosal tissue (inflammation induced by *S. typhimurium* will be discussed later).

After successful entry into epithelial cells and restoration of the epithelial cell membrane, *S. typhimurium* relies primarily on the T3SS encoded by SPI-2 to survive and replicate intracellularly by translocating SPI-2 effector proteins across the membrane of the SCV into the epithelial cell cytoplasm. SPI-2 effector proteins that appear to be necessary for survival and virulence of *S. typhimurium* inside the SCV are SifA, SseJ, SseF, SseG, SopD2, and PipB2 (50, 55, 56). SifA has been shown to promote tubulation of the SCV through correlation with another effector protein SseJ (57). SCV tubulation in conjunction with the effects of SseF and SseG localize the SCV to the perinuclear region in close proximity of the Golgi apparatus (58). The localization of the SCV is important for intracellular survival because vesicular trafficking through the Golgi network allows for the acquisition of nutrients, thereby allowing the establishment of a replication niche for *Salmonella* (58, 59).

An additional means by which SPI-2 promotes intracellular survival of *S. typhimurium* is by encoding factors that mediate the evasion of immune responses. SPI-2 promotes protection from reactive oxygen intermediates produced by macrophages, specifically nitric oxide (NO) and NADPH oxidase [for review, see Ref. (60–62)]. *S. typhimurium* has been shown to evade NO-mediated killing in macrophages by inhibiting IFN-γ-induced NO production in a SPI-2-dependent manner (61). SPI-2 is also involved in avoiding NADPH oxidase-dependent killing by interfering with the trafficking of NADPH oxidase (62). Although the specific SPI-2 effector proteins involved in the evasion of both NO-dependent and NADPH oxidase-dependent killing of *S. typhimurium* have yet to be identified, the established role of SPI-2 in evasion of both immune responses suggests a possible role for one or more encoded effector proteins in promoting resistance to reactive oxygen intermediates in macrophages.

SALMONELLA-INDUCED INFLAMMATION

IMMUNE RECOGNITION

Pathogen-associated molecular patterns (PAMPs) are recognized by pattern-recognition receptors (PRRs), namely toll-like receptors (TLRs), located on inflammatory cells and epithelial cells. TLRs can recognize a wide range of PAMPs, though some TLRs do show some specificity for particular PAMPs. For example, TLR4 is mostly involved in recognition of LPS and TLR5 is mostly involved in the recognition of bacterial flagellin. TLRs in epithelial cells are localized to the basolateral or apical membrane, as well as in intracellular vesicles. Thus, TLRs can recognize pathogens on either side of the epithelial cell monolayer and endocytosed extracellular pathogens. Additionally, inflammatory cells, such as macrophages, expressing TLRs can also recognize PAMPs. The importance of some TLRs, specifically TLR4 and TLR5, in *S. typhimurium* infection have been established, as mutating them has been shown to increase susceptibility to infection and inflammation (35). Intracellular recognition of bacteria or their products in the cytoplasm is also mediated by nucleotide-binding oligomerization domain proteins NOD1 and NOD2. NOD1 recognizes peptides containing diaminopimelic acid, which is a component of Gram-negative bacterial cell walls, whereas NOD2 recognizes a muramyl dipeptide present in the peptidoclycan layers of both Gram-positive and Gram-negative bacteria. Similar to TLRs, mutations in NOD1 and NOD2 proteins increase susceptibility to disease and infection caused by intracellular bacteria [for review, see Ref. (63, 64)].

RECRUITMENT OF IMMUNE CELLS

The host immune system also activates inflammatory pathways in response to infection with *S. typhimurium*. The binding of TLRs and NOD1/NOD2 proteins to their respective ligands activates the NF-kB pathway leading to production of pro-inflammatory cytokines and chemokines. Basolateral secretion of the cytokine IL-8 recruits neutrophils and is necessary for PMN migration into the subepithelium. Additional chemokines, such as CCL20, play a role in attracting immature dendritic cells, which upon exposure to antigen, can mature and present antigenic peptides to naïve B and T-cells in the mesenteric lymph nodes (35). *S. typhimurium* can also react with TLRs on macrophages in the subepithelial region after being transcytosed through M cells, thereby activating and inducing them to also produce cytokines and chemokines. Cytokines produced by these activated macrophages include IL-1, IL-6, and IL-23, all of which drive the differentiation of T_H17 cells whose primary function in the subepithelium is recruiting and activating neutrophils (**Figure 2**) (35, 65). Other cytokines produced by these activated macrophages include IL-18 and IL-12, both of which drive the IFN-gamma-dependent production of antigen-specific T_H1 cells (35).

MECHANISM OF NEUTROPHIL RECRUITMENT

A hallmark of *S. typhimurium*-induced inflammation is the recruitment of PMNs from the underlying microvasculature to the subepithelial region of the epithelial cell monolayer (**Figure 2**). The neutrophils then migrate across the monolayer into the lumen,

resulting in the inflammatory pathology of Salmonellosis. New information is shedding light on the molecular mechanisms and signaling pathways involved in neutrophil recruitment across the intestinal epithelium. As discussed above, it is becoming increasingly appreciated how inflammation induced by *S. typhimurium* increases its pathogenic bacterial fitness.

In addition to promoting bacterial entry, many effector proteins encoded by SPI-1 also activate inflammatory signaling pathways. The activation of Rho-GTPases by SopE, SopE2, and SopB result in the induction of mitogen-activated protein kinase (MAPK) pathways (**Figure 2**). In particular, the ERK, JNK, and p38 pathways are stimulated, resulting in the terminal activation of inflammatory regulators AP-1 and NF-κB (**Figure 2**) [Ref. (66); for review, see Ref. (50)]. Furthermore, the activation of NF-κB and AP-1 stimulates the secretion of the cytokine IL-8 on the serosal side of the epithelial cell monolayer, a requirement for the recruitment of neutrophils to the subepithelial region (**Figure 2**) (67). Although IL-8 is necessary for PMN migration into the lumen, it has been shown that IL-8 alone is not sufficient enough to drive the migration across the epithelial cell monolayer [Ref. (67, 68); for review, see Ref. (69)].

The migration of neutrophils from the basolateral side to the luminal side of the epithelial cell monolayer is driven by another PMN chemoattractant, hepoxilin A_3 (HXA_3) (**Figure 2**) (68, 70). HXA_3 is a bioactive eicosanoid that is synthesized from arachidonic acid via the 12/15-lipoxygenase pathway in epithelial cells (**Figure 2**) (70). After synthesis, HXA_3 is secreted from the apical surface of epithelial cells by an ATP-binding cassette transporter called multidrug resistant protein 2 (MRP2) (**Figure 2**) (71). Secretion of HXA_3 into the lumen forms a chemoattractant gradient that causes neutrophils to migrate from the region underlying the epithelial cell monolayer into the lumen (**Figure 2**) (70).

Activation of the effector protein SipA has been shown to be necessary for induction of HXA_3 synthesis and the resulting PMN migration (**Figure 2**) (72). Remarkably, the mechanism for activating SipA was recently shown to require processing by the host enzyme caspase-3 at a particular cleavage site, resulting in two distinct effector domains (73). Furthermore, the two domains were shown to be functionally different. The ability to promote PMN migration is confined to the SipA N-terminal domain, whereas the C-terminal domain has been shown to be involved in actin rearrangement (72, 73). The current understanding of the mechanism of SipA-dependent synthesis of HXA_3 is that SipA induces the recruitment of ADP-ribosylation factor 6 (ARF6) to the apical membrane of the epithelial cells. ARF6 activates phospholipase D, which generates phosphatidic acid. Phosphatidic acid is then converted to diacylglycerol (DAG), which recruits protein kinase C-α (PKC-α) to the apical membrane (**Figure 2**). PKC-α, in addition to an ERM protein ezrin, modulate the localization of MRP2 to the apical membrane of epithelial cells, thereby allowing the secretion of HXA_3 into the lumen and production of the chemattractant gradient that induces neutrophil transmigration (**Figure 2**) (72, 74, 75).

CONCLUSION

The architecture of the mucosal immune system, including mucins, antimicrobial peptides, resident microbiota, paracellular junctions, and effector cells of the lamina propia, functions to prevent pathogenic bacteria from disrupting the epithelial cell monolayer and causing disease. If enteric pathogens are able to penetrate these barriers, then it results in a host inflammatory response and eventually activation of an adaptive immune response, designed to eradicate the intruding pathogen. However, *S. typhimurium* has evolved systems, namely the SPI-1 and SPI-2 T3SS, to manipulate the defensive mechanisms of the mucosal immune system in order to develop a replication niche in the mucosal epithelium. Additionally, the ability of *S. typhimurium* to exploit inflammation allows it to penetrate the epithelial barriers, a condition in which activation of the adaptive immune response would be required for pathogenic clearance. Investigating how *S. typhimurium* exploits host cell signaling pathways will allow for increased understanding in its pathogenesis, and consequently provide further insight into how inflammation can seemingly result in both increased bacterial fitness and increased pathogenic clearance.

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Mucosal-associated invariant T-cells: new players in anti-bacterial immunity

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Mucosal-associated invariant T (MAIT) cells are an innate-like T-cell population involved in anti-bacterial immunity. In human beings, MAIT cells are abundant, comprising ~10% of the $CD8^+$ T-cell compartment in blood. They are enriched at mucosal sites and are particularly prevalent within the liver. MAIT cells are defined by the expression of a semi-invariant T-cell receptor (Vα7.2-Jα33/12/20) and are restricted by the non-polymorphic, highly evolutionarily conserved MHC class Ib molecule, MHC-related protein (MR)1. MR1 has recently been shown to present an unstable pyrimidine intermediate derived from a biosynthetic precursor of riboflavin; riboflavin biosynthesis occurs in many bacteria but not in human beings. Consistent with this, MAIT cells are responsive to riboflavin-metabolizing bacteria, including *Salmonella*. In mouse models, MAIT cells have been shown to play a non-redundant role in anti-bacterial immunity, including against *Escherichia coli*, *Klebsiella pneumoniae*, and *Mycobacterium bovis* BCG. In human beings, MAIT cells are decreased in frequency in the blood of patients with tuberculosis or pneumonia, and their frequency has been inversely correlated with the risk of subsequent systemic bacterial infection in patients in intensive care. Intriguingly, MAIT cells are also depleted from the blood early in HIV infection and fail to recover with anti-retroviral therapy, which may contribute to the susceptibility of patients infected with HIV to certain bacterial infections, including non-typhoidal *Salmonella*. In this review, we will discuss what is currently known about MAIT cells, the role that *Salmonella* has played in elucidating MAIT cell restriction and function, and the role MAIT cells might play in the control of *Salmonella* infection.

Keywords: MAIT cells, anti-bacterial, inflammation, CD161, TCR, MR1

INTRODUCTION

In 1999, Tilloy et al. first described mucosal-associated invariant T (MAIT) cells (1). Interest in this unique subset of innate-like T-cells has increased rapidly over the last 5 years as novel findings have revealed their unique anti-bacterial function and phenotype (2, 3). MAIT cells represent the most abundant innate-like T-cell population within human beings, comprising up to ~5% of the total T-cell population; this compares with just ~0.1% for invariant natural killer T (iNKT) cells (4, 5). They are characterized by the expression of a semi-invariant TCR (Vα7.2-Jα33/12/20) that recognizes the evolutionarily conserved MHC-like protein 1 (MR1), which presents a bacterial-derived ligand (6–11). Although they can be activated through their TCR, they are also readily stimulated by innate cytokines, either leading to the expression of pro-inflammatory cytokines or the release of cytotoxic and pro-inflammatory granzymes (**Figure 1**) (12, 13). Furthermore, MAIT cells have been associated with a number of disease settings, including bacterial infections (14), and pro-inflammatory diseases such as multiple sclerosis (15) and psoriasis (16). Thus, this large T lymphocyte population is likely to have an important role in human health.

This review will explore what is currently known about MAIT cells in human beings. Comparisons between human and murine MAIT cells have been made elsewhere (4). Furthermore, we will discuss the role that *Salmonella* has played in identifying the functions of this cell type, and the potential role MAIT cells may have in controlling *Salmonella* infections.

MAIT CELL PHENOTYPE

In addition to possessing the Vα7.2-Jα33/12/20 TCR, MAIT cells can be identified in human beings by the expression of a characteristic phenotypic signature composed of a number of additional surface and transcriptional markers.

MEMORY PHENOTYPE

In adults, MAIT cells typically express an effector memory phenotype: $CD45RO^+$, $CCR7^-$, $CD62L^-$, $CD27^+$, and $CD28^+$ (17–19). However, in cord blood, MAIT cells possess a naïve phenotype ($CD45RA^+$, $CCR7^+$, $CD62L^+$), but still retain a phenotypic signature characteristic of adult MAIT cells, including the expression of CD161, interleukin (IL)-18Rα, CD8αα, and CCR6 (3, 5, 17, 20). A recent study demonstrated that MAIT cells in the thymus, spleen, and mesenteric lymph nodes of aborted second trimester fetuses also had a naïve phenotype and expressed only low levels of the characteristic MAIT cell markers, such as IL-18Rα and CD8αα, while MAIT cells in the fetal intestine, liver, and lung had a more memory phenotype (21).

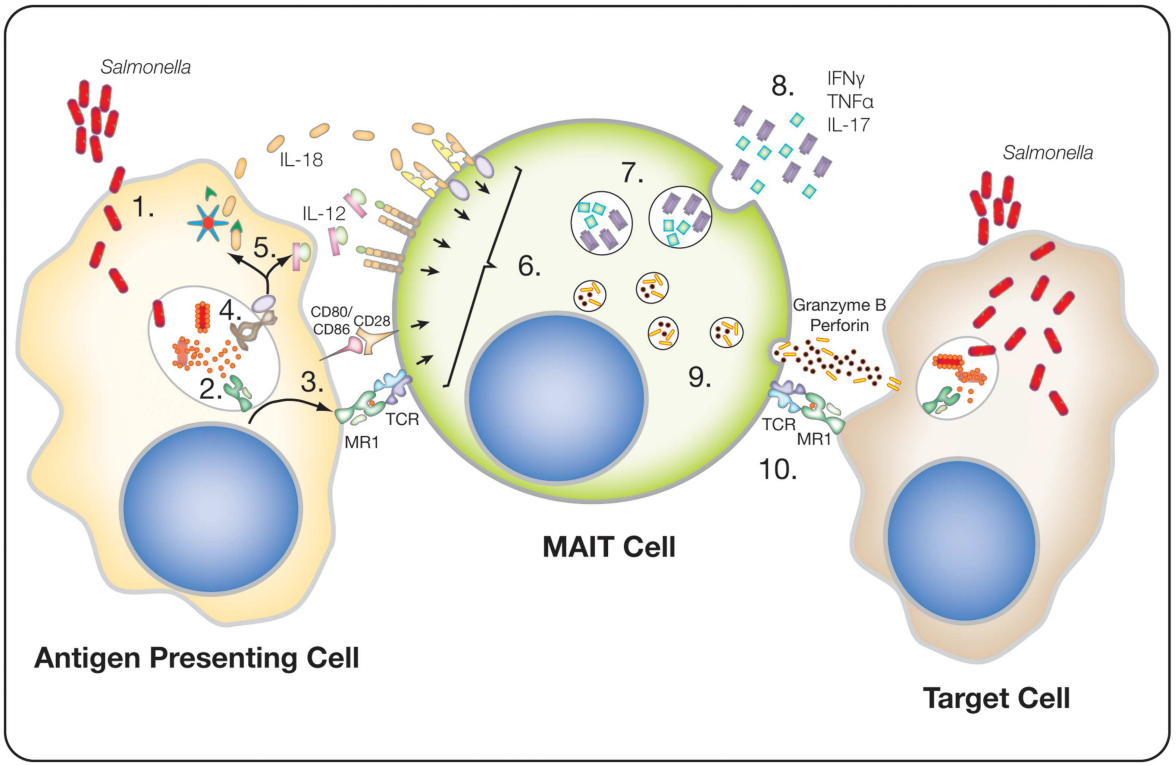

FIGURE 1 | Potential MAIT cell response to *Salmonella* infected cells. **(1)** Internalization of *Salmonella* by an antigen-presenting cell, either through infection or actively by phagocytosis. **(2)** Lysis of the bacteria, within endocytic compartments, releases 5-A-RU, which is converted to 5-OE-RU or 5-OP-RU and binds to and stabilizes MR1. **(3)** The stable MR1 translocates to the cell surface, where it is presented along with other co-stimulatory molecules, e.g., CD80 or CD86. **(4)** Bacterial components trigger pathogen recognition receptors (PRR), such as TLR8. **(5)** PRR triggering drives cytokine expression, such as IL-12, and the activation of the inflammosome, resulting in the release of active-IL-18. **(6)** MAIT cells are activated either by TCR recognition of MR1 in combination with co-stimulatory receptors, e.g., CD28, and/or by cytokines, e.g., IL-12 and IL18. **(7)** Activated MAIT cells express pro-inflammatory cytokines, e.g., IFNγ, TNFα, and IL-17. **(8)** These cytokines can directly act anti-bacterially, or recruit and stimulate other immune cells, e.g., neutrophils by IL-17. **(9)** Activation of MAIT cells upregulates perforin and granzyme B expression. **(10)** Theoretically, the degranulation of cytotoxic granules into infected cells (target cells), via recognition of MR1, could induce cell death and, thus, the potential clearance of infected cells.

CD161

CD161 is a C-type lectin-like receptor originally identified by Lanier et al. (22). It is found on a broad range of lymphocytes, including $CD4^+$, $CD8^+$, $\gamma\delta^+$ T-cells, and NK cells. The majority of NK cells express CD161 (>90%), while in the $CD4^+$, $CD8^+$, and $\gamma\delta^+$ T-cell subsets, CD161 expression is limited to ~30% of cells (19, 23). However, within the $CD8^+$ and $CD8^-$ $CD4^-$ T-cell population, CD161 expression can distinguish three separate subsets, $CD161^-$, $CD161^{intermediate/+}$, and $CD161^{high/++}$; MAIT cells populate the $CD161^{++}$ subset (17, 18). In adult peripheral blood, MAIT cells represent ~85% of the $CD161^{++}$ subset (24). However, in cord blood, the MAIT cells make up a much smaller proportion of this subset, averaging ~15% of the $CD161^{++}$ $CD8^+$ T-cell population (21, 25, 26). During early childhood, this population expands so that by the age of 24 months the MAIT cell population already represents ~50% of the $CD161^{++}$ $CD8^+$ T-cell population (25).

The function of CD161 on MAIT cells is yet to be fully elucidated. On NK cells, binding of CD161 to its ligand [lectin-like transcript (LLT) 1] leads to an inhibition of cytotoxicity (27–29). Two studies explored the role of CD161 on $CD8^+$ T-cells and reached opposing conclusions (27, 29). Rosen et al. found that cross-linking CD161 had no effect on anti-CD3/CD28 stimulated $CD8^+$T-cells in terms of IFNγ expression and inhibited TNFα expression, whereas Aldemir demonstrated increased IFNγ expression after CD161 signaling. Le Bourhis et al. recently reported that ligation of CD161 on MAIT cells inhibited cytokine production but had no effect on cytotoxicity (30). One explanation for these differences could be due to the different CD161 cross-linking antibody clones used.

CD161 surface expression has recently been reported to be downregulated after long-term activation *in vitro*; downregulation was associated with greater rounds of proliferation (13). In HIV infection, loss of expression of CD161 by MAIT cells has been suggested (31). Given that MR1-tetramer-positive cells are not found in the CD161-negative population in healthy individuals (8), this requires further investigation.

IL-18Rα

CD161 expression is associated with high levels of IL-18Rα expression. Of all human T lymphocytes, MAIT cells express the highest level of IL-18Rα (12, 17). Naïve MAIT cells, derived

from cord blood, have also been shown to be IL-18Rα$^+$ (5, 24). Recent observations of tissue from second trimester fetuses suggest that IL18Ra expression is low upon egress from the thymus but increases as MAIT cells mature in the tissues (21). Expression of IL-18Rα conveys an ability to respond robustly to cytokine stimulation (discussed below), which is limited in the other human T-cell populations (12).

ZBTB16

The development of MAIT cells parallels that of NKT cells. Both innate-like T-cell subsets are selected for by non-classical MHC molecules, MR1 and CD1d, respectively, expressed on double negative thymocytes (32), and both subsets also express the transcription factor ZBTB16 (33). However, while NKT cells exit the thymus as an expanded and mature population, MAIT cells do not. Instead, murine models have shown that MAIT cells within the periphery have a naïve and immature phenotype, lacking expression of ZBTB16, CD25, CD69, or ICOS and further require an established microbiota and B-cells to expand into a memory phenotype (33). In contrast, MAIT cells found in the periphery of human fetuses have already acquired a more mature, memory phenotype, expressing ZBTB16, IL-18Rα and, upon bacterial stimulation, the capacity to express IFNγ and, in cells from the small intestines, IL-22 (5, 21). What drives selection and maturation *in utero* is unclear, but suggests that an endogenous ligand for MR1 may exist. Alternatively, this may reflect *in utero* exposure to commensal microflora, as has recently been suggested (34–36).

RORγT

CD161 expression is also a key phenotypic maker of IL-17 secreting cells (19, 37). Within the CD4$^+$T-cell subset, Th17 cells represent a novel helper cell population that can secrete IL-17A under the control of the master transcription factor retinoic acid-related orphan receptor (ROR) γt (38, 39). Within the CD8$^+$T-cell subset, the expression of RORγt and the secretion of IL-17 are restricted to Tc17 cells that are CD161^{++}. Thus, MAIT cells represent the vast majority of Tc17 cells (17, 24). The ability of MAIT cells to express IL-17A has recently been shown to depend on their pre-exposure to cytokines IL-1beta, IL-23, and IL-7 (40). Interestingly, hepatic stromal cells constitutively express IL-7, emphasizing the link between MAIT cells and the liver (41, 42).

CO-RECEPTOR USAGE

CD161^{++}/MAIT cells also encompass the CD8alpha/alpha population in adult blood, small bowel, and liver (5). Interestingly, this population is not seen in cord blood, or fetal thymus, but is seen at low levels in fetal intestine, liver, and lung (21), suggesting that it is derived from the CD8alpha/beta population through the regulation of CD8beta. Functionally and phenotypically, there is no difference between the CD8alpha/alpha and CD8alpha/beta subsets of the CD161^{++}/MAIT cell population (5). However, Walker et al. have also described the expression of CD8alpha/alpha as the marker of terminally differentiated CD8$^+$ T-cells that can be found in a number of chronic viral infections (26).

MULTIDRUG RESISTANCE TRANSPORTER ABCB1

Mucosal-associated invariant T-cells express the ATP-binding cassette (ABC) B1 drug resistance transporter, which can also be seen on CD161$^+$T-cells to a more limited degree (43). The ability to efflux drugs has been shown to allow MAIT cells to persist during chemotherapy for the treatment of acute myeloid leukemia (AML) or breast cancer (24, 43). The expression of the ABCB1 transporter possibly reflects the diverse environmental toxins MAIT cells are exposed to in their niche, although its overall role is not defined.

CHEMOKINE RECEPTOR EXPRESSION

Mucosal-associated invariant T-cells express a range of chemokine receptors (CCR6, CCR5, CCR9, and CXCR6) that localize them to the gut, but more prominently to the lungs and liver (24, 44). The liver receives 25% of its blood supply from the hepatic artery and 75% from the portal vein. The portal vein delivers blood direct from the gastrointestinal track and spleen, placing the liver in the front line in the defense against microbial infection. Moreover, Balmer et al. recently described the liver as a firewall against infection from commensal bacteria that have invaded the body through either the intestines or systemic vascular circuits (45). Therefore, given their anti-bacterial function, it is unsurprising that MAIT cells represent up to 45% of all liver lymphocytes (24, 40). Furthermore, both CD161$^+$ CD4$^+$ and CD161$^+$CD8$^+$ T-cells are selectively recruited to the liver during inflammation (17, 23, 40).

ANTI-BACTERIAL ACTIVITY OF MAIT CELLS

Two studies in 2010, by Gold et al. and Le Bourhis et al., observed that MAIT cells could recognize antigens derived from a range of bacteria presented on antigen presenting cells (APCs) (2, 25). Gold et al. observed that a large population of CD8$^+$ T-cells able to respond to *Mycobacterium tuberculosis* (Mtb) was present even in unexposed individuals. Functional and phenotypic analysis showed that this population was MR1 restricted and expressed the TCR Vα7.2 chain, i.e., MAIT cells. Furthermore, they showed that MAIT cells responded to *Salmonella enterica* serovar Typhimurium-infected dendritic cells, as well as *Escherichia coli* and *Staphylococcus aureus*. Le Bourhis et al. demonstrated the ability of MAIT cells to recognize and be activated by monocytes exposed to *E. coli* in an MR1-dependent manner. Furthermore, they observed that MAIT cells are activated by a wide range of bacteria (*E. coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Lactobacillus acidophilus, Staphylococcus aureus, Staphylococcus epidermidis,* and *Mycobacterium abscessus*) but not by all (*Enterococcus faecalis, Streptococcus pyogenes*). In addition, they showed activation in response to some yeasts (*Candida albicans, Candida glabrata,* and *Sacchromyces cerevisiae*). Moreover, MAIT cells were not observed in germ-free mice or in germ-free mice repopulated with *E. faecalis*, but were in germ-free mice repopulated with *Enterobacter cloacae* or *L. acidophilus*, underscoring the important role of certain bacterial species in MAIT cell development.

The ability of MAIT cells to control bacterial infections was elegantly demonstrated by Georgel et al. through the use of luminescent-*K. pneumoniae* (46). Despite the low frequency of MAIT cells in common laboratory strains of mice, they showed that in the absence of MR1, and, therefore, MAIT cells, mice succumbed to disseminated infection, while wild-type mice cleared the infection within 2 days. No defect in clearance was seen with *E. coli, Shigella dysenteriae,* or *Yersinia enterocolitica* suggesting that redundancy in the immune response is sufficient to control the

dissemination of these organisms. In contrast, enhanced control of *E. coli* infection and *M. abscessus* infection was demonstrated in Vα19 or Vβ6 transgenic mice, which have an increased frequency of MAIT cells, on a MR1 replete background compared with a $MR1^{-/-}$ background. Subsequent studies by Chua et al. and Meierovics et al. have demonstrated the need for MAIT cells in the early control of *Mycobacterium bovis*, BCG, and *Francisella tularensis* following a mucosal challenge (47, 48). Moreover, the presence of MAIT cells had a strong influence on the timing of recruitment and activation of conventional T-cells, and provided long-term protection alongside a conventional T-cell response (48).

THE MHC-RELATED PROTEIN (MR)1 AND ITS LIGAND

The broad range of bacteria MAIT cells can respond to was recently explained with the identification of the ligand that binds MR1 (49). Kjer-Nielsen et al. discovered the structure of MR1 and the nature of the ligand that it binds after their serendipitous finding that 6-formyl pterin (6-FP), a photodegradation product of folic acid that was present in tissue culture media, was able to bind to MR1 and allowed its refolding. However, while 6-FP was able to stabilize the MR1 molecule, it was unable to activate MAIT cells. Given that the culture supernatant of *S. enterica* serovar Typhimurium was able to activate MAIT cells, they reasoned that it must contain the activating ligand. Therefore, they refolded MR1 with the culture supernatant of *S. enterica* serovar Typhimurium grown in minimal media that lacked vitamins (M9 minimal media), and analyzed the refolded MR1 by mass spectrometry. They observed a single ligand with a mass to charge ratio (*m/z*) of 329.11. This was consistent with the ligand being reduced 6-hydroxymethyl-8-D-ribityllumazine (rRL-6-CH2OH), a metabolic by-product of riboflavin metabolism. Chemical synthesis of reduced rRL-6-CH2OH confirmed that it had the same *m/z* as the ligand identified in the culture supernatant. Furthermore, synthetic reduced rRL-6-CH2OH, as well as related compounds derived from riboflavin metabolism, 7-hydroxy-6-methyl-8-D-ribityllumazine (RL-6-Me-7-OH) and 6,7-dimethyl-8-D-ribityllumazine (RL-6,7-diMe), were able to activate primary MAIT cells. This pivotal discovery was consistent with the prior observation that MAIT cells could be activated by *Salmonella*, along with other *Enterobacteriaceae*, *P. aeruginosa*, *S. aureus*, and some yeast species, which all contain the riboflavin synthetic pathway, but not by *S. pyogenes* or *E. faecalis*, which lack the riboflavin synthetic pathway (2, 25).

As the origin of the previously identified ligand, rRL-6-CH2OH, was not clear, Corbett et al. derived various strains of *Lactococcus lactis* with different mutations in the riboflavin synthesis operon and assessed their ability to activate MAIT cells (50). Mutants lacking genes involved in the riboflavin synthesis pathway (*ribA* and *ribG*) were unable to activate MAIT cells, and the MR1-binding ligand (*m/z* 329.11) was undetectable. By contrast, no defect in MAIT cell activation was seen with *ribB* and *ribH* mutants, pinpointing the production of 5-amino-6-D-ribitylaminouracil (5-A-RU), an early intermediate in riboflavin synthesis. The importance of 5-A-RU was confirmed by the lack of MAIT cell activation and absence of the MR1-binding ligand (*m/z* 329.11) in the culture supernatant of *S. enterica* serovar Typhimurium SL1344 with mutated *ribD* and *ribH* genes; furthermore, complementation restored reactivity. Despite the necessity for 5-A-RU, it was unable to bind MR1 or activate MAIT cells directly. However, upon non-enzymatic condensation with glyoxal or methylglyoxal, byproducts of other metabolic pathways (including glycolysis), 5-A-RU formed unstable intermediates [5-(2-oxoethylideneamino)6-D-ribitylaminouracil (5-OE-RU) and 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU)], which were able to covalently bind to MR1. Furthermore, these unstable intermediates formed reversible covalent Schiff base complexes with Lys43, analogous to 6-FP (49). Moreover, 5-OE-RU and 5-OP-RU could be demonstrated in the culture supernatant of activating bacteria, but not of non-activating bacteria, and could be captured by MR1. Therefore, MR1 captures unstable intermediates that would otherwise convert to lumazines. MR1 refolded with 5-A-RU and methylgyoxal (5-OP-RU) produced a mass spectrometry peak of *m/z* 329.11, consistent with what was originally found in *Salmonella* supernatant (49). This suggested that 5-OP-RU, and not reduced rRL-6-CH2OH, was the true ligand for MR1. In support of this hypothesis, they also found a 315.09 *m/z* species (corresponding to 5-OE-RU) bound to MR1 refolded in the presence of *E. coli* DH5α; the 315.09 *m/z* species was also detected with *Salmonella*, but was much less abundant. This interesting finding demonstrates that different bacteria may produce different MAIT-activating ligands.

Overall, our current understanding of this complex process is that bacterially derived 5-A-RU is converted to 5-OE-RU or 5-OP-RU by reaction with bacterial or host-derived metabolites. These unstable intermediates covalently bind to, and stabilize MR1, which can then be recognized by the MAIT cell TCR.

Recently, Eckle et al. published that MAIT cells activation can be competitively inhibited by a novel MR1 ligand, acetyl-6-formylpterin (Ac-6-FP) (51). Ac-6-FP was identified in a search for analogs of 6-FP that were stronger antagonists of MAIT cell activation. MR1 efficiently refolded in the presence of Ac-6-FP and increased surface expression was seen in an MR1-transduced cell line treated with Ac-6-FP. Neither Ac-6-FP-MR1 tetramers nor 6-FP-MR1 tetramers were able to stain PBMCs. Consistent with antagonist activity, Ac-6-FP strongly inhibited the activation of the Jurkat.MAIT cell line in response to the agonist ligands, rRL-6-CH2OH and 5-OP-RU. Therefore, Ac-6-FP will be a useful tool in future studies of MAIT cell function.

THE MAIT CELL TCR

The defining feature of MAIT cells is the expression of a semi-invariant TCR, Vα7.2Jα33/12/20, which restricts MAIT cells to the evolutionarily conserved, non-polymorphic MHC-related protein 1 (MR1) (1, 6, 9, 11, 44). Vbeta chain usage does vary; however, it is dominated by Vβ2 and Vβ13.2 (1, 9, 11). Homologous TCRs have also been identified in mice (Vα19Jα33, paired with Vβ6 or Vβ8) as well as cattle (1, 5, 52).

The structure of the MAIT cell TCR and the residues that are critical for interacting with ligand-bound MR1 were determined prior to identification of the MAIT cell ligand (9, 11). Analysis of the MAIT cell TCR structure demonstrated that the CDR3α region is composed of the Vα7.2 and Jα33 of the alpha chain. *S. enterica* serovar Typhimurium was used as the source of the MR1-binding ligand to show individual residues in Vα7.2 (in CDR1α Gly28α

and Asn30α, in CDR2α Tyr48α, and in CDR3α Asp92α, Asn94α, and Tyr95α), but not the Vβ chain, made critical contacts with MR1. However, switching the entire CDR3β loop with one from a non-MAIT cell TCR abolished recognition of MR1. A similar pattern was observed with other bacteria (*E. coli, K. pneumoniae, P. aeruginosa,* and *S. epidermidis*). Given the wide specificity, the authors suggested that the MAIT cell TCR was like a pattern recognition receptor. By mutational analysis, they identified residues Leu65 and Glu158 on MR1 as critical for the interaction with the TCR. These two residues mapped centrally to opposite sides of the antigen-binding cleft of MR1.

The identification of the ligand paved the way for the development of MR1 tetramers for the identification of MR1-restricted T-cells (8) . Reantragoon et al. used MR1 with a K43A mutation to make tetramers. MR1 K43A could be refolded in the absence of ligand and once refolded was able to be loaded with reduced rRL-6-CH2OH. To further prove that the MR1-tetramer was specific to MAIT cells, PBMCs were depleted of MR1-tetramer binding cells and tested for reactivity to *S. enterica* serovar Typhimurium supernatant or to reduced rRL-6-CH2OH.

Using the loaded tetramers, Reantragoon et al. confirmed that the majority of MR1-restricted T-cells express the canonical semi-invariant T-cell receptor, Vα7.2-Jα33, but also identified minor (8–31%) MAIT cell populations exist that express Vα7.2-Jα12 and Vα7.2-Jα20. Interestingly, all alpha chains contained the conserved Tyr95α within CDR3α loop, which forms a hydrogen bond with RL-6-Me-7-OH bound by MR1 and is crucial for MAIT cell activation (8, 53). Interestingly, non-canonical TCRs showed a bias in Vβ usage toward TRBV6-4. Both canonical and non-canonical TCRs appeared to have the same ligand specificities. MR1-tetramer staining of lymphocytes isolated from the jejunal mucosa confirmed that MAIT cells are enriched at this site, with ~60% of $CD3^+$ CD4-cells being MAIT cells. Furthermore, the majority of the tetramer positive cells in the jejunum were shown to be Vα7.2-Jα33, as in blood. This suggests that frequency of MAIT cells varies in different anatomical locations within the gastrointestinal tract, with higher frequencies in jejunum (8) than in ileum (54), colon, and rectum (31, 55). Alternatively, the variations observed in MAIT cell frequency in different tissues may reflect differences in methodology between studies.

Using a mouse MR1 tetramer in a MAIT-enriched mouse model (transgenic for Vα19 on Cα−/− background), the differences between mouse MAIT cells and human MAIT cells were highlighted (8). While human MAIT cells were predominantly $CD8^+$ with some double negative cells and only a small $CD4^+$ subset, in Vα19-transgenic mice >40% of MAIT cells were $CD4^+$, with the remainder mostly DN rather than $CD8^+$. Whether this reflects functional differences between human and mouse MAIT cells remains to be determined.

A recent study by Gold et al. investigated the TCR usage of MAIT cells that were responsive to different microbes (56). $CD8^+$ T-cells from healthy donors were stimulated *ex vivo* with A549 cells infected with *Mycobacterium smegmatis, Salmonella typhimurium,* or *C. albicans.* Vα7.2 + CD8 + T-cells that produced TNFα were sorted and their TCR usage determined. They found greater diversity in the *TRAJ* gene usage than previously reported; while *TRAJ33* dominated, a range of other *TRAJ* genes were identified, including some (*TRAJ9* and *TRAJ39*) that do not encode for the Tyr95a residue that has been reported to be critical for MAIT cell activation (8, 11, 52). However, as the authors note, there is no allelic exclusion at the *TRA* locus, so the non-canonical *TRAJ* genes identified may not contribute to the functional MAIT cell TCR. *TRAJ* usage and CDR3α sequence of the responding MAIT cells differed between microbes; there was more similarity in the CDR3α sequence in MAIT cells activated by the same microbe than with those activated by a different microbe. Furthermore, there was significant diversity in *TRBV* gene sequences of MAIT cells responding to different microbes, although there was minimal overlap in the CDR3β sequence across donors and microbes. Overall, this suggested that different MAIT cells respond to different microbe-derived ligands. In support of this, of four MAIT cell clones that were robustly activated by *M. smegmatis*, only two were activated by RL-6,7-diMe. The authors speculate that MR1 ligand diversity drives MAIT cell TCR diversity and that that the Vα chain primarily mediates contact with MR1, while the CDR3β chain, positioned above the MR1 ligand-binding groove, contributes to ligand discrimination, as previously described by López-Sagaseta et al. (57, 58). This is consistent with the findings of Eckle et al., who showed that the novel inhibitory MR1 ligand, Ac-6-FP, induced structural alterations in MR1, which prevented the MAIT cell TCR CDR3β chain from binding (51). These two studies open up the exciting possibility that a wide range of novel MR1 ligands exists, which could modulate the MAIT cell response.

MAIT CELL ACTIVATION

A number of studies have suggested adult MAIT cells to be terminally differentiated and, as a result, less responsive to TCR signaling, showing low IFNγ production and little proliferation compared to stimulation that bypasses the TCR (such as activation by PMA and ionomycin, or PHA) (24, 33, 43). In contrast, MAIT cells derived from cord blood or fetal tissues readily proliferate with TCR stimulation (21, 33). This lack of responsiveness by adult MAIT cells to TCR stimulation can, however, be overcome. Turtle et al. demonstrated that TCR signaling required the addition of an innate signal/s from either the co-receptor CD28 or the cytokine IL-12 in order to induce high levels of IFNγ and strong proliferation (20). Using transcriptional profiling, they went on to show that the mechanisms controlling TCR signaling in MAIT cells were distinct from those seen in anergic or exhausted T-cells.

A study by Chua et al. demonstrated that in a murine model of bacterial infection using *M. bovis* BCG, IL-12 signaling, but not TCR signaling, was required for the control of infection; blockade of IL-12 but not MR1, inhibited the anti-bacterial activity of MAIT cells (47). This regulation of activation is similar to that seen in iNKT cells, where IL-12 signaling dominates over CD1d-induced TCR signaling (59, 60). In human models of MAIT cell activation, there is a dual role for TCR- and cytokine-induced activation of MAIT cells. At early time points, 5 hours after a MAIT cell encounters an antigen-presenting cell (APC) presenting its cognate antigen on MR1, TCR signaling dominates activation (12). However, at later time points (20 hours), cytokine-mediated activation is equally important and MR1 blockade has a more limited effect. In contrast to the murine models, IL-12 alone is not sufficient to induce IFNγ expression, but requires the addition of other

innate cytokines, such as IL-18 (12). The ability to respond to IL-12 plus IL-18 is similar to that of NK cells (61), and implicates MAIT cells in a range of infectious and non-infectious inflammatory diseases. Furthermore, signaling via toll-like receptors (TLR) is able to drive the expression of a range of pro-inflammatory cytokines from professional APCs, which can activate MAIT cells (**Figure 1**) (12, 62). We have shown that TLR8 agonists are particularly potent stimulators of IL-12 and IL-18 secretion, and therefore capable of driving MAIT cell IFNγ expression (12, 62). Thus, this suggests that in addition to their anti-bacterial role, MAIT cells may play an important role in anti-viral responses, and this may also provide a mechanism that explains their involvement in other pro-inflammatory settings such as experimental autoimmune encephalomyelitis, multiple sclerosis, inflammatory bowel disease, psoriasis, and arthritis (15, 16, 54, 63, 64).

MAIT CELL CYTOTOXICITY

How MAIT cells affect their anti-bacterial function remains poorly defined. Upon activation, MAIT cells are able to produce several cytokines, including IFNγ, TNFα, and IL-17 (17, 24). In addition, it has recently been demonstrated that MAIT cells are also cytotoxic. Le Bourhis et al. reported that MAIT cells can recognize epithelial cells (HeLa cells) infected with *Shigella flexneri* but not *S. enterica* serovar Typhimurium (30). Consistent with this, MAIT cells were able to kill HeLa cells infected with *S. flexneri* but not *S. enterica* serovar Typhimurium. This cytotoxicity was dependent upon MR1 and was evident in HeLa cells expressing endogenous levels of MR1. No reactivity was seen with a *Salmonella* pathogenicity island 1 (SpI-1)-deleted strain of *S. enterica* serovar Typhimurium (SpI-1 is required for invasion). Similarly, MAIT cell activation and cytotoxicity was not seen with a SpI2-deleted strain of *S. enterica* serovar Typhimurium. Therefore, the virulence factors that prevent MR1 loading remain to be defined. The authors' suggestion was that *Salmonella* avoids detection as it resides in vacuoles and prevents fusion with the lysosome, while *Shigella* escapes to the cytoplasm. Indeed, invasion by *Shigella* was important as HeLa cells infected with a DMxiD strain were unable to efficiently activate MAIT cells. *In vivo* activation (determined by HLA-DR expression) and decreased frequency of MAIT cells in blood was observed in human beings after oral challenge with an attenuated strain of *S. dysenteriae* suggesting that MAIT cells responded to *S. dysenteriae in vivo.*

Recently, Kurioka et al. described increased cytotoxic potential of MAIT cells after activation (13). These cytotoxic, or "licensed," MAIT cells displayed upregulation of granzyme B, normally not expressed in resting MAIT cells, and enhanced perforin expression, which is low in resting MAIT cells (13). The licensed MAIT cells displayed an increased capacity to kill cognate-target cells, and maintained this phenotype even after several rounds of proliferation.

A ROLE FOR MAIT CELLS IN THE CONTROL OF *SALMONELLA* INFECTION

The role of MAIT cells in *Salmonella* infection remains to be defined. As discussed above, MAIT cells can be activated by *Salmonella* sp. *in vitro* (8, 11, 25). MAIT cells activated by *Salmonella* sp. produce IFNγ and TNFα; these cytokines have been shown to be important in the control of *Salmonella* infection (65). In addition, MAIT cells can secrete IL-17 in response to stimulation with *E. coli* (55). IL-17 has recently been suggested to be critical in preventing the dissemination of *Salmonella.* In IL-17 receptor-deficient mice, increased systemic dissemination of *S. enterica* serovar Typhimurium was seen (66). Furthermore, in SIV-infected rhesus macaques, increased dissemination of *S. enterica* serovar Typhimurium was seen, which was associated with loss of Th17 cells and the IL-17 response from the ileum (66). Therefore, along with the loss of Th17 cells (67), the loss of MAIT cells in HIV infection may contribute to the increased susceptibility to disseminated non-typhoidal *Salmonella* infection (68).

Mucosal-associated invariant T-cells may also contribute to the control of *Salmonella* infection through cytotoxic activity. Cytotoxic T-cells are important in the clearance of *Salmonella* infection (69). However, MAIT cells are unable to kill HeLa cells, an epithelial cell line, infected with *Salmonella* (69). This may be because live *Salmonella* sp. is able to avoid MR1 containing compartments by preventing phagosome-lysome fusion (70). Future investigations with live *Salmonella* sp. may help elucidate the MR1 loading pathway/s, and subsequent activation of MAIT, revealing potential therapeutic targets.

The low numbers of MAIT cells in common laboratory mouse strains has hampered the study of the role MAIT cells in response to *Salmonella* infection. Therefore, a robust murine model is required to investigate the role of MAIT cells in the control of *Salmonella* infection.

MAIT CELLS IN INFECTIOUS DISEASE

The role of MAIT cells in human disease has not been fully assessed due to the difficulties of obtaining tissue samples. However, there are a number of interesting associations between the frequency of MAIT cells within peripheral blood and disease. During active *M. tuberculosis* infection, MAIT cells numbers are lower in peripheral blood compared to healthy controls (2, 25, 71, 72). Consistent with these findings, Grimaldi et al. looked at MAIT cell numbers in critically ill septic and non-septic patients (14). They observed that all critically ill patients studied, including those with severe bacterial or viral infections and those with non-infective illness, had low MAIT cell counts compared to healthy controls. This was least pronounced in individuals with severe viral infections, and most striking in individuals infected with bacteria other than *Streptococcus* species (14). This suggests that the loss of MAIT cells from the periphery could be due, in part, to compartmentalization during disease (2, 25). Interestingly, however, the authors also observed that those individuals with persistent MAIT cell depletion at day 4 post-admission were at increased risk for subsequent nosocomial infections. Therefore, MAIT cell exhaustion or death, two mechanisms proposed to occur in HIV (see below), may contribute to this phenotype.

In both HCV and HIV, MAIT cells are depleted from the blood (17, 31, 55). During HCV infection, the loss of peripheral MAIT cells is potentially due to their relocation to the liver (17). Moreover, the frequency of IFNγ and IL-17 dual-expressing $CD8^+$ T-cells in the liver, a proxy for MAIT cells, was inversely correlated with the fibrosis score, suggesting that they either play a protective role during HCV infections, or that they are progressively lost

from the liver with increasing fibrosis. This might contribute to the higher rates of bacteremia seen in individuals with cirrhosis (45, 73, 74).

In HIV infection, the loss of $CD161^{++}$ MAIT cells from blood occurs early during infection and persists despite otherwise successful anti-retroviral therapy (31, 55, 71, 75, 76), although the nature of this perturbation is unclear. Cosgrove et al. reported that MAIT cells, defined as $CD161^{++}$ $CD8^{+}$ T-cells by flow cytometry, were depleted as a proportion of the $CD8^{+}$ T-cell population in blood (55). They proposed that this depletion was due, at least in part, to activation induced cell death from overstimulation secondary to microbial translocation. While Leeansyah et al. also observed a decrease in size of the $CD161^{++}V\alpha7.2^{+}$ population, they suggested that this was due to downregulation of CD161 and functional exhaustion of MAIT cells (31). In support of this, they noted an increase in frequency of CD161-$V\alpha7.2^{+}$ T-cells as a proportion of $CD3^{+}$ T-cells. Importantly, the antibody against Vα7.2 used in these studies is not specific for the canonical MAIT cell TCR (33). Therefore, the recently described MR1 tetramer will be useful to determine whether the CD161-$V\alpha7.2^{+}$ T-cells seen in HIV infection are MAIT cells or not (8). Interestingly, in healthy donors, MR1 tetramer does not bind the CD161-$V\alpha7.2^{+}$ T-cell population.

MAIT CELLS IN INFLAMMATORY DISEASE

In addition to their anti-microbial activity, MAIT cells have also been implicated in a range of pro-inflammatory settings. Serriari et al. observed that individuals with inflammatory bowel diseases had lower numbers of circulating MAIT cells compared to controls (54), as seen during bacterial and viral infections (2, 17, 25, 54, 71). However, within individuals, the frequencies of MAIT cells were increased within inflamed tissues compared to healthy tissue, suggesting recruitment of MAIT cells from the blood to sites of inflammation. This is a theme consistent in other inflammatory diseases such as psoriasis, rheumatoid arthritis, multiple sclerosis, and experimental autoimmune encephalomyelitis (15–17, 63, 77). Interestingly, $CD56^{-}$ MAIT cells have been observed infiltrating kidney and brain tumors, implying a potential role in tumor immunity (78).

Overall, these studies in human disease demonstrate that MAIT cells are a population of innate-like cells that rapidly translocate to sites of inflammation, regardless of whether the inflammation is due to bacterial infection or to other pro-inflammatory stimuli.

WHAT THE FUTURE HOLDS FOR MAIT CELL RESEARCH

Through the use of tools such as *Salmonella*, our understanding of MAIT cell functions has increased rapidly over the last 5 years. However, there are still a number of important questions to be answered. Are there other ligands presented by MR1? Is there an endogenous ligand within the thymus for MAIT cell selection? How is MR1 regulated? What are the relative roles of TCR-dependent and TCR-independent triggering of MAIT cells in host defense? Understanding what MR1 presents and how it is regulated will be critical for understanding where and when MAIT cells have a definitive role in disease. What role MAIT cells play during human disease, in both infectious and autoimmune settings also needs to be addressed. Although it is important to study human disease, much will be learnt from animal models. Understanding how MAIT cells are regulated will potentially allow their *in vivo* manipulation for a positive outcome. Given the rise of antibiotic resistant bacteria, as highlighted by the recent WHO report (April 2014) future prophylactic and therapeutic strategies that harness the anti-bacterial potential of MAIT cells may be particularly important (79, 80).

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Salmonella as a model for non-cognate Th1 cell stimulation

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Salmonella has been a model pathogen for examining CD4 T cell activation and effector functions for many years due to the strength of the Th1 cell response observed during *Salmonella* infections, the relative ease of use of *Salmonella*, the availability of *Salmonella*-specific T cell reagents, and the well-characterized nature of the model system, the pathogen, and the immune response elicited. Herein, we discuss the use of *Salmonella* as a model pathogen to explore the complex interaction of T cells with their inflammatory environment. In particular, we address the issue of bystander activation of naïve T cells and non-cognate stimulation of activated and memory T cells. Further, we compare and contrast our current knowledge of these non-cognate responses in CD8 versus CD4 T cells. Finally, we make a case for *Salmonella* as a particularly appropriate model pathogen in the study of non-cognate CD4 T cell responses based on the strength of the Th1 response during infection, the requirement for CD4 T cells in bacterial clearance, and the well-characterized inflammatory response to conserved molecular patterns induced by *Salmonella* infection.

Keywords: *Salmonella*, CD4 T cell, Th1 cell, innate stimulation, TLR, NLR, IFN-γ

INTRODUCTION

T cell activation and effector functions have been extensively studied *in vitro*, allowing for controlled interactions within a defined environment. However, studying T cells *in vitro* inherently limits interactions to those that have been previously defined. To explore more complex systems of interactions beyond known parameters requires using an *in vivo* model system. One common technique for studying T cell responses *in vivo* is to examine a population of T cells with known antigen specificity. This includes the use of T cell receptor (TCR) transgenic mice, model antigens like ovalbumin, and major histocompatibility complex (MHC) class I and II tetramers presenting defined peptide sequences, which allows for the detection of T cells specifically recognizing that peptide. These reagents have greatly facilitated the tracking of antigen-specific T cells and the study of monoclonal T cell responses. Together with *in vitro* studies, the examination of antigen-specific T cells *in vivo* has been essential in defining much of what we know about T cell immunology.

When trying to understand the diverse polyclonal responses that are induced by infections, *in vivo* techniques that examine individual antigen-specific responses are likely to be limited. The natural breadth of the naïve TCR repertoire is an important strength of the adaptive immune response and can only be maintained by having pools of individual clones at very low frequency. Recent evidence has shown that altering the frequency of a given T cell clone can impact the activation strength, kinetics, and memory formation of the resulting T cell response (1–4). This issue complicates TCR transgenic mouse studies, which focus on a monoclonal population, generally used at unnaturally high frequency. Studying the natural endogenous precursor population is therefore important and also complex since the frequency of individual clones also varies within the naïve repertoire (5).

Furthermore, individual TCR specificities may be predisposed toward different fates (6) and may also be regulated by temporal and anatomical antigen expression by the pathogen, factors that might significantly affect some clonal populations differently than the overall polyclonal T cell response (7, 8). These issues affect the use of TCR transgenic mice, MHC tetramer studies, and model antigens, because it may lead to a situation where the T cell response under study may not be representative of the overall T cell response to the pathogen. Likewise, studies that attempt to activate T cells with model antigens in the absence of infection are unlikely to accurately reflect the complex interactions that occur between T cells and the rest of the immune system in the context of a strong inflammatory response. Thus, to examine the full range of T cell functions and interactions within the larger immune network, it is necessary to study them in the context of a natural polyclonal response that includes a broad range of antigens and the inflammatory milieu that differentiates infection from other surrogate means of activation.

When exploring the responses of CD4 T cells, in particular, it is critical to examine their functions under circumstances in which they are naturally induced and required. In other words, it makes very little sense to study the effector function of Th1 cells using models where these Th1 cells do not contribute to pathogen clearance. The role of the Th1 subset of CD4 T cells and its effector cytokine IFN-γ in *Salmonella* infections has been very well established (9–11), making *Salmonella* model systems particularly appropriate for characterizing Th1 cell functions. Additionally, the innate immune response and inflammatory responses occurring during *Salmonella* infections are relatively well-defined (12–16), making it an ideal model to characterize the influence of natural inflammatory conditions on these Th1 cell responses.

In this review, we will highlight the unique advantage of the *Salmonella* model system for studying Th1 responses to innate stimuli. First, in Section "Armed and Ready: T Cell Responses to Innate Signals," we discuss and compare conventional cognate T cell stimulation, non-cognate stimulation of activated conventional T cells, and the responses of innate-like T cells. Thus far, most studies examining non-cognate T cell responses have focused on CD8 T cells, primarily in viral infection models. It is likely that the rules governing non-cognate CD8 T cell responses differ in certain aspects to those governing non-cognate responses in CD4 T cells. However, comparing these responses in infection models that generate overall weak CD4 T cell responses due to poor activation does not allow accurate comparison of the capacity of the non-cognate CD4 T cell response. In Section "A Complicated Relationship: The Dynamic Interactions of *Salmonella* and Th1 Cells," we discuss the dynamic interaction of *Salmonella* and the Th1 response, focusing on the important role of Th1 cells in the resolution of *Salmonella* infection, and potential ways that *Salmonella* might be able to subvert cognate T cell recognition and thus increase the requirement for non-cognate recognition pathways.

ARMED AND READY: T CELL RESPONSES TO INNATE SIGNALS

CONVENTIONAL T CELL RESPONSES

Before discussing innate stimulation of T cells, we will first briefly review initial T cell activation, differentiation, secondary stimulation at sites of infection, and formation of memory. Both CD4 and CD8 T cells are activated upon recognition of a specific peptide sequence in host MHC by the TCR. CD8 T cells recognize this peptide presented within MHC-I expressed on most cell surfaces, while CD4 T cells interact with antigen presented in MHC-II only by antigen presenting cells (APCs). These APCs, often dendritic cells (DCs), collect and process antigen in the periphery and return to the lymph nodes and other secondary lymphoid organs with the antigens presented on their surface, where they can be recognized by interacting T cells. This first antigen-specific interaction is referred to as signal 1 of T cell activation (**Figure 1**) because, although it is required, the TCR stimulation alone is not sufficient to functionally activate the T cell. On its own this TCR interaction will ultimately lead to anergy, a state of unresponsiveness that maintains peripheral tolerance.

During the initial TCR:MHC interaction, the T cell requires a second signal for the priming process. This second signal is referred to as costimulation (**Figure 1**), and can be achieved by a number of different interactions, although the most common activating signal is between CD28 on CD4 T cells and B7 molecules on DCs. The expression of these costimulatory molecules are upregulated on DCs after DC activation by inflammation, increasing the likelihood that T cells will be activated only when the antigen is encountered by the DC under appropriate conditions. While this second signal prevents anergy, a third signal is required to complete CD4 T cell priming and instruct the differentiation pathway.

Differentiation is a critical step in CD4 T cell priming because of the eclectic capacities of CD4 T cells. Once a CD4 T cell has recognized antigen presented by MHC-II and a costimulation signal, a third cytokine signal (**Figure 1**) will instruct the CD4 T cell to differentiate into a subset trained for a particular function. In this review, we focus on the Th1 subset of CD4 T cells, in which interleukin-12 (IL-12) allows sustained upregulation of the transcription factor T-bet and, upon re-stimulation, production of effector cytokines such as IFN-γ, TNFα, and IL-2. Additional CD4 subsets include Th2 (which respond to IL-4 and are regulated by GATA3), Th17 (combinations of TGF-β, IL-6, IL-21, and IL-23 lead to RORγt expression), Tfh (T follicular helper, respond to IL-6 and IL-21 to upregulate Bcl-6), and iTreg cells (induced T regulatory, TGF-β and IL-2 lead to expression of Foxp3), as well as other, less well-characterized subsets of CD4 T cells. It is important to note, however, that substantial evidence now supports the impermanence of some of these differentiation pathways, a concept known as effector plasticity. Thus, while CD4 T cells require these initial differentiation instructions, they often retain the capacity to acquire new functions under sufficient alternative stimulation.

The initial process of T cell activation dramatically alters the cell, causing upregulation of cascades of transcription factors, as well as altering the miRNA regulation, epigenetic modifications, and post-translational pathways. These changes program the cell with the capacity to rapidly respond upon re-stimulation in a specialized manner. However, the actual secretion of effector cytokines still requires some regulation to prevent unnecessary inflammation. For this reason, activated T cells arrive at sites of infection primed for rapid response, but not constitutively secreting cytokines. Traditionally, the secondary interactions of activated T cells at sites of infection are believed to consist of additional antigen-dependent interactions of the TCR and peptide-MHC

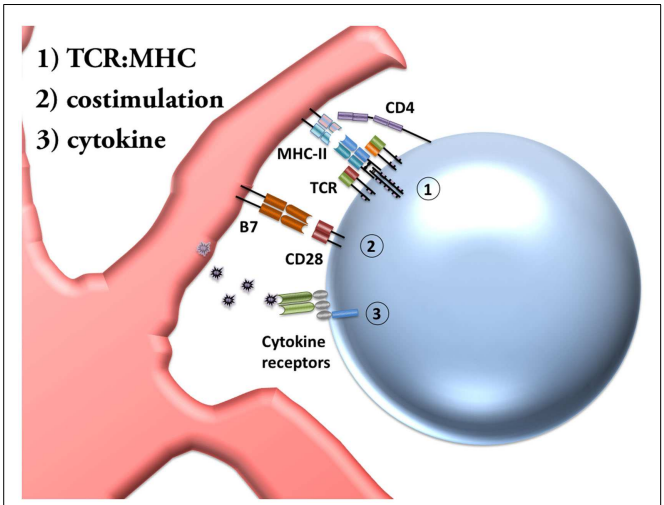

FIGURE 1 | Priming of CD4 T cells requires three signals. Conventional activation of naïve CD4 T cells requires three distinct signals. Signal 1: the TCR (T cell receptor) on the T cell must recognize a particular peptide sequence, processed within an APC (antigen presenting cell) and presented by MHC-II (major histocompatibility complex-II) in mice. The CD4 co-receptor shown stabilizes the T cell interaction with MHC-II. Signal 2: activated DCs (dendritic cells) upregulate the costimulatory molecules B7.1 and B7.2 (also called CD80 and CD86). CD28 on T cells recognize these costimulatory molecules as a second signal for activation. In the absence of costimulation T cells undergo anergy or death. Signal 3: the cytokine environment instructs the final stage of T cell priming by determining the differentiation pathway undergone by the activated T cell.

complexes, which triggers a transient robust effector response in the appropriate location (17). Unlike the initial activation process, antigen-specific interaction alone is sufficient to induce cytokine production, because of the T cell's activated state (18). However, much of this work has been conducted in low-inflammatory conditions and focused on the requirement for TCR interactions. Such work does not rule out a role for inflammatory stimulation of T cells during infections. Indeed, the ability of inflammatory cytokines to either activate naïve T cells or stimulate cytokine secretion from effector T cells will be discussed in the next section.

After initial activation, T cells undergo massive clonal expansion. This expansion of specific effector Th1 cells typically takes a few days, and in prolonged infections like *Salmonella* this T cell response can take a few weeks to reach the peak of expansion. During this time, T cells are responding to a complex network of signals, including IL-7 to survive, IL-2 to proliferate, pro-inflammatory cytokines, anti-inflammatory cytokines, and potentially secondary TCR signals. The combination of these encounters does more than just stimulate T cells to produce effector cytokines – it establishes their fate. While T cell responses are critical to pathogen clearance in many cases, they also have the potential to damage host tissues and for this reason, most T cells will ultimately be instructed to die. Thus, after the peak of clonal expansion T cells undergo a contraction phase in which most T cells receive apoptotic signals and are removed from circulation.

However, some T cells receive just the right combination of stimuli and survival signals to transition from an "armed and ready" effector state to a quiescent memory state. CD4 T cells can exist in a number of different memory states, which may ultimately affect their longevity, the areas in which they circulate, and the requirements for re-activation. The best described examples of CD4 memory subsets are the central versus effector memory T cells, which circulate in lymphoid tissue or non-lymphoid tissue, respectively. CD8 T cells are also believed to form these subsets, as well as memory populations called short-lived effector cells (SLECs) or memory precursor effector cells (MPECs) whose formation depends heavily on the inflammatory signals received, but which have not been described for CD4 T cells. Memory T cells are an important component of the rapid response to re-challenge with previously encountered pathogens because of their lowered activation threshold, pre-differentiated state, and extensive epigenetic modifications that allow for rapid relay of the signals needed to elicit effector function. Understanding how these memory T cells are formed and are able to respond is especially crucial to vaccine design.

While the above mechanisms comprise a very basic understanding of conventional T cell activation, there are a number of caveats and exceptions that are worth discussing. In Section "Bystander Activation and Non-Cognate Stimulation," we will discuss the ability of T cells to respond to non-TCR stimuli, focusing on what has been referred to in the literature alternatively as bystander activation or non-cognate stimulation. A partial mechanism for non-cognate stimulation of Th1 cells is illustrated in **Figure 2**. In Section "Innate-Like T Cells and ILCs," we will outline some of the non-conventional T cell subsets that are able to respond to non-cognate stimuli to draw parallels between these "innate-like" cell types and the innate-like functions of classically activated T cells. **Figure 3** compares the interactions that occur in each of these cell responses and highlights areas that require further elaboration.

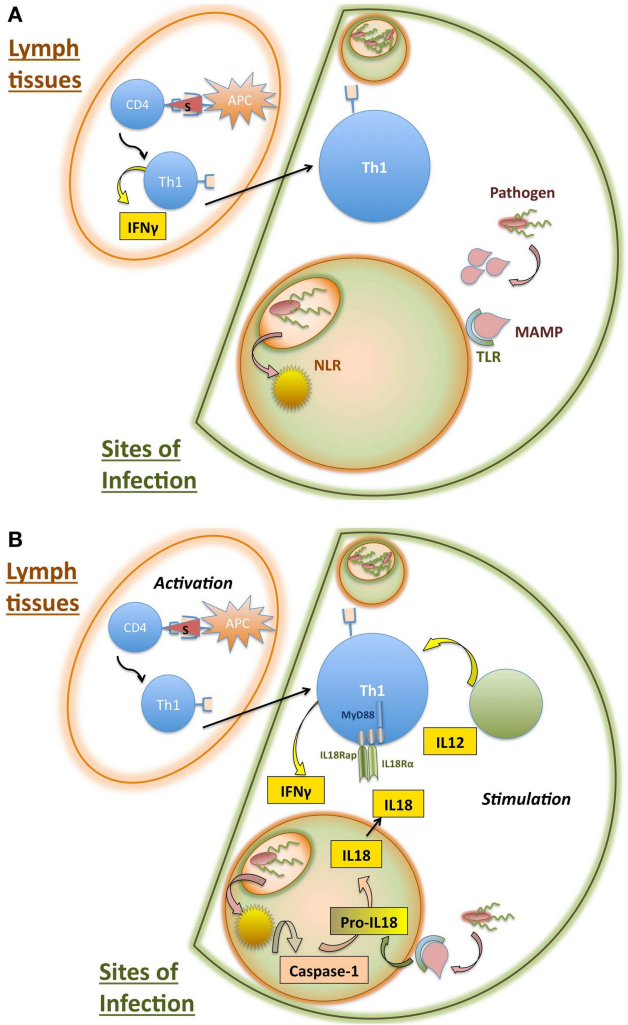

FIGURE 2 | Partial mechanism for non-cognate stimulation of activated CD4 T cells. **(A)** Initiation of innate and adaptive immune responses during intracellular infections. CD4 T cells are initially activated in lymph tissues upon recognition of particular peptide antigens presented by antigen presenting cells (APCs). During intracellular infections, the presence of IFN-γ and IL-12 results in differentiation of these activated CD4 T cells into Th1 cells, which produce IFN-γ. The Th1 cells then traffic to sites of infection, where they require additional stimulation to induce production of effector cytokines. Meanwhile, sites of infection experience inflammation elicited by innate recognition of pathogens. Pattern recognition receptors such as toll-like receptors (TLRs) and nod-like receptors (NLRs) recognize conserved products of infection, called microbe-associated molecular patterns (MAMPs). **(B)** Innate inflammation stimulates Th1 cells to amplify effector response at sites of infection. TLR recognition of innate ligands results in the upregulation of pro-IL-18, while NLR recognition of infection activates caspase-1. Caspase-1 cleavage of pro-IL-18 into the mature form of the cytokine IL-18 allows secretion. IL-18 receptor is required by Th1 cells for non-cognate elicitation of IFN-γ. CD4 T cell stimulation at sites of infection likely involves additional cytokine pathways, including IL-12, which can act synergistically with IL-18 to stimulate Th1 cells.

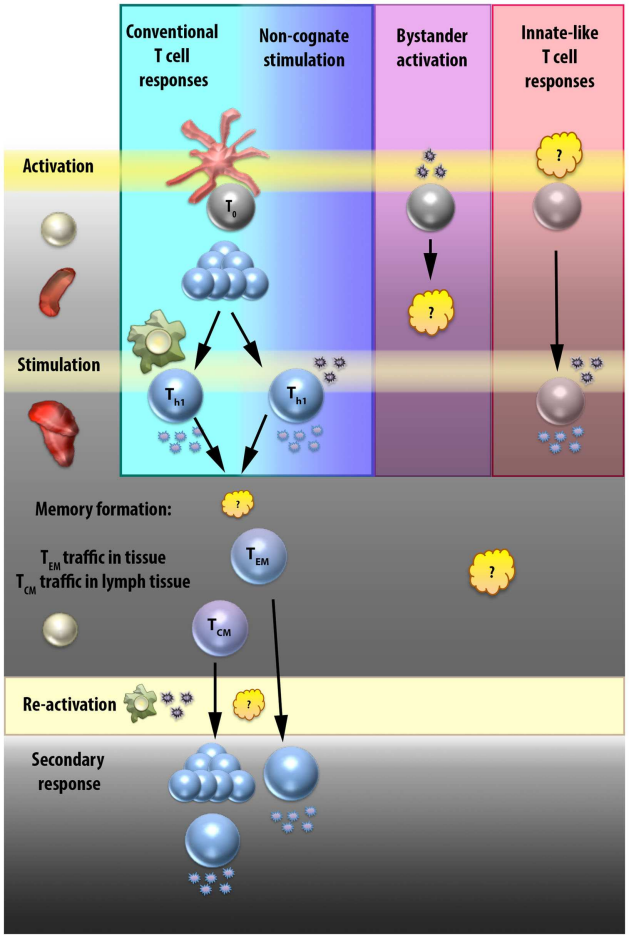

FIGURE 3 | Elicitation of CD4 and "innate-like" T cell effector functions. T cell effector functions can result from interactions with APCs or cytokines at various stages. In the conventional T cell response (first column, blue), naïve T cells are activated by direct interactions with APCs in the secondary lymphoid tissues, proliferate, migrate to sites of infection, and then are stimulated by secondary direct APC contact to produce effector cytokines. Some of these effector T cells will go on to become memory T cells. T central memory (T_{CM}) cells circulate in secondary lymphoid tissues, and upon re-activation will once again proliferate and differentiate into effector cells. T effector memory (T_{EM}) cells can either migrate in the periphery or be resident in tissue, and respond more rapidly than the T_{CM} cells because upon re-activation they can secrete effector cytokines directly. The signals that result in the T_{EM} or T_{CM} fate decision are still unclear, as are the interactions required by each for re-activation, although it is presumed that re-activation occurs after direct interaction with an APC. Non-cognate stimulation (second column, indigo) occurs at sites of infection in T cells that have already undergone conventional activation in the secondary lymphoid tissues. Instead of being stimulated by secondary direct APC contact, these cells receive stimulatory signals from cytokines that induce IFN-γ production. Whether these cells go on to join the memory pool and how this different stimulation signal affects the fate decision of CD4 T cells is unknown. Bystander activation is a term that has been used loosely to mean any TCR-independent T cell stimulatory interaction. In the third column (purple), we focus on the idea of bystander activation as a mechanism to prime a CD4 T cell in a TCR-independent manner. While the effect of cytokines on naïve T cells have been studied at length *in vitro*, there is limited evidence that a naïve CD4 T cell can be activated by cytokine signals alone, and no evidence that TCR-independent activation

(*Continued*)

FIGURE 3 | Continued
can produce a fully functional effector T cell. The last column (pink) provides a general representation of innate-like T cell subsets. Although the initial priming signals for the different innate-like T cell populations vary and are still unclear in some cases, they include alternative activation mechanisms such as restricted TCRs or constitutive priming. Stimulation of effector responses at sites of infection in innate-like T cells is known to occur rapidly in response to cytokine stimulation, hence the name "innate-like" T cells. However, it is possible that all T cells have the capacity, once activated, to respond rapidly to cytokine stimulation, and that what really separates these innate-like cell types are their unique priming mechanisms.

BYSTANDER ACTIVATION AND NON-COGNATE STIMULATION

The strict processes governing conventional T cell activation are important to avoid the uncontrolled activation of effector T cell responses. However, under some circumstances, such as during a rapidly dividing or systemic infection, these may become a hindrance to achieving the necessary strength and rapidity of the effector response. Thus, non-cognate stimulation of conventional T cells has been described in a number of model systems. Non-cognate interactions are defined negatively as any stimuli without TCR recognition of cognate peptide-MHC complexes presented on APCs. This type of T cell activation has often been referred to as "bystander activation" (19–22). This name seemingly refers to the idea that these are T cells which just happen to be in proximity to inflammatory stimuli that is perhaps intended for other cognate T cells, thus, assuming that non-cognate stimulation is an accidental "bystander" to the conventional response. However, it is equally possible that this "bystander" response is not incidental, but instead represents an integral functional capacity of T cells to respond and recognize inflammatory stimuli that are produced under extreme stress.

The earliest descriptions of bystander activation focus on cytokine or innate stimuli that drive T cell proliferation in the absence of antigen (20, 21, 23). These innate stimuli are also referred to as microbe-associated molecular patterns (MAMPs), and include toll-like receptor (TLR) and NOD-like receptor (NLR) ligands among others. However, it should be noted that most of this early work was completed in viral infection models, focuses largely on CD8 T cells, and does not differentiate between naïve and previously activated T cells. Thus, it is difficult to conclude from these studies whether naïve T cells can actually be primed by non-cognate interactions, particularly naïve CD4 T cells. Further, these data must be interpreted with caution, since cytokine-induced proliferation may not lead to an effector T cell state, and especially given the evidence that these same signals can induce apoptosis (20). Transient expression of the early activation marker CD69 was observed in naïve CD8 after Type I IFN stimulation, but neither this activation was not maintained nor was it shown to induce effector functional capacity (21).

In addition to non-cognate proliferation or upregulation of activation markers, there is also considerable work describing elicitation of effector functions from CD8 T cells by non-cognate stimuli, which is also confusingly referred to as bystander activation or stimulation (19, 24, 25). However, it is important to note that this work generally describes stimulation of previously activated T cells, or makes no distinction between activated and

naive T cells, thus, this is not activation in the sense of initial T cell priming. For clarity, in **Figure 3**, a non-cognate primary activation interaction is illustrated as "bystander activation," while a non-cognate interaction following (but separate from) a cognate primary activation interaction is referred to as "non-cognate stimulation." This stimulation of activated T cells is discussed further below.

While most of the work described above examines CD8 T cells, less examination of TCR-independent effector responses exists specific to CD4 T cells, and most shows that bystander responses occur only in previously activated or memory CD4 T cells (26–28). There are, however, limited *in vitro* data showing that very high doses of IL-2 can make naïve CD4 T cells responsive to IL-12 or IL-18 without TCR signaling (29). Considering these distinctions, as well as the inability of many of these studies to rule out innate-like populations that were not yet identified at the time the studies were conducted, the question of whether naive T cells can be primed in a non-cognate manner under sufficient inflammatory stimulation, and whether these cells would be functional as effectors *in vivo*, lacks a definitive answer. However, non-cognate stimulation of effector and memory T cells has been shown, and some of the mechanisms are beginning to be understood.

As described in the previous section, an activated Th1 cell is primed to produce effector cytokines rapidly. Here, we focus on the effector cytokine IFN-γ, secreted by both CD8 T cells and Th1 cells. This priming means that the IFN-γ gene locus is modified to be open for faster transcription, IFN-γ mRNA has been transcribed and is ready for rapid translation, and IFN-γ protein has been translated and awaits the signals necessary for post-transcriptional modifications and secretion. Despite all of this preparation, Th1 do not constitutively secrete IFN-γ. When they receive antigen-specific TCR signals, the signaling cascade downstream of the TCR allows rapid release of the prepared IFN-γ transcripts and protein from the regulatory mechanisms that otherwise keep this production in check. However, other signaling pathways can also stimulate IFN-γ production in activated Th1.

The signals eliciting production of IFN-γ in various cell types have been studied extensively, and yet the complexity of the regulation of this cytokine continues to unfold. For example, while T-bet is considered to be a master transcriptional regulator of IFN-γ production, in natural killer (NK) cells and CD8 T cells Eomes is able to make up for the loss of T-bet, while in CD4 T cells T-bet signaling is required (30). Further, it was shown that the promoter region of IFN-γ that is utilized after TCR signaling is different than the promoter region required for IFN-γ production in response to IL-12 and IL-18 (31). In addition to transcriptional regulation, extensive post-transcriptional regulation of IFN-γ mRNA has been shown to take place, and varies between naïve, effector, and memory T cell populations (32).

While some evidence has suggested that an ability for innate ligands to interact directly with CD4 T cells to stimulate their proliferation or function (33), it is also likely that innate ligands can stimulate T cells through a second messenger that would allow amplification of the signal. Many cells are capable of responding to TLR or NLR ligands to produce inflammatory cytokines, including those cytokines known to stimulate IFN-γ production from Th1 cells. Thus, it is easy to imagine a mechanism whereby Th1 cells respond indirectly to innate stimuli by responding to inflammation. In fact, this has been shown to occur during viral infections in response to TLR ligands, and during *Salmonella* infection in response to NLR ligands, both in CD8 T cells (24, 25, 34).

Recently, we described a mechanism for innate Th1 stimulation that relies on the convergence of both TLR and NLR signaling pathways to elicit IL-18 production, which can then be recognized by activated Th1 to result in IFN-γ secretion. This mechanism is illustrated in **Figure 2**. Previous work has typically focused on very small populations of innately responding T cells, particularly for CD4 cells, consequently supporting the concept of the "unintended bystander." However, the small numbers of T cells that can be seen responding at any given time during the normal course of infection do not necessarily represent a small subset of cells capable of innate response. Nor does this imply that innate stimulation of T cells occurs too infrequently to have a significant impact. Using *Salmonella* infection, we showed that Th1 cells in an infectious model of strong Th1 activation are highly susceptible to innate stimulation, with a large proportion of the Th1 capable of secreting IFN-γ in response to LPS stimulation. Finally, we demonstrated that mice whose T cells lack the capacity to be innately stimulated by IL-18 suffer a reduced capacity to clear *Salmonella* infection (35). Together, this suggests that a pathway of innate T cell response that not only can occur but must also occur for normal immune function.

INNATE-LIKE T CELLS AND ILCs

While the previous section focused on the non-cognate interactions of conventional αβ T cells, numerous cell types have been described that have overlapping surface markers, developmental lineages, transcription factor profiles, or effector functions to conventional T cell subsets, but which respond in a non-conventional manner (36). These cells are often thought of as innate-like cells with adaptive-like functions that can provide critical assistance in the early immune response. Examples include natural killer T (NKT) cells, mucosal-associated invariant T (MAIT) cells, γδ T cells, and innate lymphoid cells (ILCs).

Innate lymphoid cells are a rapidly expanding group of cells defined predominantly by their lack of lymphocyte antigen receptors (TCR or BCR, B cell receptor) or lineage-specific markers (36). Recently, a uniform nomenclature for ILCs was proposed that divides the various cells into three main groups. Much like the CD4 T helper subsets, Group 1 ILCs can be characterized by the expression of T-bet and IFN-γ, Group 2 express GATA3, and Group 3 express RORγt and produce IL-17 or IL-22 (37). Most ILCs require IL-7R signaling and express the surface marker CD90 and the transcription factor ID2, although it has been proposed in mice that IL-7 may inhibit transition of ILC3 to ILC1 (38). Further elaboration will center on the Group 1 ILC subset, given its similarity to the Th1 subset of CD4 T cells.

Natural killer cells have been included within this nomenclature as a Group 1 ILC, alongside ILC1. While NK cells have been very well-described as an early source of IFN-γ and TNFα, in addition to their cytotoxic functions, very little is known about ILC1. Although there is evidence that they develop from ILC3s after IL-12 stimulation, it was recently shown that ILC1 can also develop independently from a common innate lymphoid progenitor (39).

However, whether they arise separately or as a consequence of functional plasticity, it remains that there is a group of T-bet+ ILCs, which can respond to IL-12 and IL-18 signals to produce IFN-γ, but which are not NK cells and do not exhibit cytotoxicity (37, 38). As a whole, the early innate effector responses of the Group 1 ILCs during intracellular infections play a key role in host protection, inflammation, and initiation of adaptive responses.

While ILCs lack a TCR, several other cell types express unique TCRs that allow for a non-conventional response, known collectively as "innate-like T cells" due to their ability to respond rapidly to innate stimulation. Among these innate-like T cells, one common method allowing for a non-cognate-antigen response is an invariant or semi-invariant TCR. While conventional TCRs undergo recombination activating gene (RAG) dependent rearrangement of their α and β chains during development in the thymus to allow for a broader repertoire with improved specificity, some innate-like cell populations possess TCRs with single α-chain and restricted β-chain specificities. Particular examples of cells with these alternative TCRs include invariant NKT cells (iNKT) and MAIT cells (36).

Natural killer T cells are perhaps the best described of these innate-like T cell subsets. NKT cells are innate-like T cells in the sense that they develop in the thymus and express a TCR, but they also express NK1.1 and several innate activating or inhibitory receptors typically found on NK cells (40). Two types of NKT cells exist: NKT I are the well-described, iNKT cells known to respond to lipids and especially with high affinity to α-galactosylceramide presented by the MHC-related molecule CD1d, while NKT II are less studied, to date, but have a diverse TCR repertoire and fail to respond to α-galactosylceramide (41). While iNKT can be activated by CD1d presentation of foreign lipid antigens, they may also be activated by CD1d presentation of lipid self-antigens and require inflammatory cytokine signals, allowing for more rapid and innate-like responses (40–42). Further, it was recently shown that iNKT can be activated in an antigen-independent manner by cytokine alone during some infections, like *Salmonella*, but not others (43).

Another semi-invariant T cell population are the recently described MAIT cells, characterized by their localization to mucosal tissue and their recognition of the MHC-related molecule, MR1, which binds the metabolites of B vitamins generated by bacteria and fungi (36). MAIT cells develop and are pre-programed in the thymus, but quickly acquire an activated phenotype in the periphery. There is now evidence to suggest that this activation occurs in response to microbiota; in particular, the observation that germ-free mice have diminished numbers of MAIT cells, which can be recovered upon monoculture reconstitution with many bacteria or yeast, but not *Enterococcus faecalis*, which lacks the riboflavin metabolic pathway. Unlike NKT, they seem to respond predominantly to TCR ligation and do not require cytokine stimulation to elicit effector functions, which consist mostly of IFN-γ and TNFα, although they can also express IL-17 (44, 45).

γδ T cells are a unique exception, in that they possess recombined TCRs, but can respond in a rapid, innate-like manner to inflammatory cytokines. Thus, these T cells are technically a component of the adaptive immune response, but are often discussed in the context of early innate responses (46). Differentiation programing of γδ T cells occurs during thymic development, determining either an IFN-γ, IL-17, or IL-4 producing phenotype (47), but peripheral activation is still required before effector functions can be elicited. The relative contributions of TCR, costimulation and cytokine signals to this activation still seem to be a matter of some debate, and may be partially dependent on the subset, but whatever the mechanism these cells respond far more rapidly than their αβ T cell counterparts (46).

All of these innate-like T cells (iNKT, MAIT, and γδT), although possessing different TCRs and recognizing different antigen repertoires, share some common features. For one thing, in each cell type the ability to generate or maintain immunological memory is poorly defined, as are the required signals for survival and proliferation (36). For both iNKT and γδ T cells functional subsets have now been described analogous to the CD4 T helper subsets (40, 47), although unlike CD4 T cells these subsets are pre-determined during development in the thymus. Although subsets have not yet been defined as such for MAIT cells, and they typically respond to IL-12 to produce IFN-γ in a T-bet-dependent manner, they also express RORγt and can express IL-17 and IL-22 under appropriate stimulation. Further, there is now some evidence for an immunoregulatory function of MAIT cells. Thus, whether MAIT cells have functional subsets or are simply functionally promiscuous remains to be determined (44, 45).

In further similarity, each is described as "innate-like" due to an ability to rapidly respond to innate stimulation – that is, they respond to the inflammatory cytokines that result from innate stimuli. However, for each cell subset the specific requirements of initial priming, and in particular whether this priming can occur without any peripheral TCR stimulation, is still a matter of debate within their respective fields. While earlier literature suggested that these cells respond rapidly because they are able to respond to cytokine alone, other work shows that these cells require TCR interactions (48), and for iNKT at least this requirement can be met by self-antigens under inflammatory conditions to allow more rapid responses (40–42). More recent evidence suggests that these requirements may differ under varying circumstances (43). Herein, we make an argument that conventional αβ T cells can also respond rapidly to inflammatory cytokines in an innate-like manner once they have been primed. The parallels between these responses and their mechanisms suggest that a conservation of these stimulatory mechanisms between conventional and innate-like T cells, and highlight the need for a better understanding of the activation requirements of non-conventional T cells.

A COMPLICATED RELATIONSHIP: THE DYNAMIC INTERACTIONS OF *SALMONELLA* AND Th1 CELLS

SALMONELLA: A PERSISTENT GLOBAL CHALLENGE

While many bacteria live and replicate extracellularly, entering the host cell only when engulfed and destroyed by phagocytes, some bacteria have adapted unique survival strategies to allow a protected life cycle within host cells. Some of these bacteria are obligate intracellular pathogens, like *Chlamydia*, that cannot replicate outside of the cell, but many intracellular bacteria are capable of occupying either space. The immune system has, in turn, developed a

number of ways to recognize pathogens within cells, pathways which the pathogen actively attempts to thwart (49).

In this review, we focus on *Salmonella*, a Gram-negative enteric pathogen that resides predominantly within the phagosomes of macrophages located in the spleen, liver, and bone marrow. In human beings, there are two forms of systemic salmonelloses: typhoid fever and non-typhoidal salmonellosis, or NTS. Typhoid and paratyphoid fevers are caused by the human-specific pathogens *Salmonella enterica* serovar Typhi and Paratyphi, still occur endemically in developing countries, and can cause severe systemic disease even in healthy individuals. Estimates range as high as 27 million annual infections with either Typhi or the clinically indistinguishable Paratyphi. In contrast, NTS occurs only in immunocompromised individuals, but can originate from any of the >2000 *Salmonella* serovars capable of causing foodborne illness in human beings and harbored in a wide variety of animal reservoirs (50). Thus, both systemic infections remain a source of concern for public health officials worldwide.

Although antibiotics effective against *Salmonella* are available, the options are relatively limited for intracellular pathogens as compared to more accessible extracellular pathogens. Additionally, among those antibiotics currently available, there is a growing incidence of drug resistance, including multi-drug resistance to the first-line treatments, and resistance to the now standard fluoroquinolones. Further, decreased susceptibility to the fluoroquinolone ciprofloxacin has been associated in enteric fever patients with prolonged fever and increased rates of treatment failure (51). Finally, an analysis of the *Salmonella* metabolic pathways has suggested that most of the major or non-redundant pathways have already been targeted or considered for drug inhibition, suggesting that a limitation to prospective future development of new antibiotic treatments (52). Together, these studies emphasize the need for alternative treatment options and for improved vaccination strategies that could lessen the need for, and consequently the selective pressure upon, traditional antibiotic therapy.

Currently, two vaccinations are commercially available in the U.S. for travelers to typhoid endemic countries. One is a Vi capsular polysaccharide (ViCPS) vaccine administered intramuscularly as one dose at least 1 week prior to exposure. The second is an oral, attenuated Ty21a vaccine available under several formulations, typically administered every other day as three separate doses 2 weeks prior to exposure. Both vaccinations suffer from limitations that impair their practicality in typhoid endemic regions, not the least of which is the need for regular re-vaccination, and the low-reported efficacy at 3 years of 51–55% (53). The ViCPS vaccine is approved in children over the age of 2 years old, and the oral vaccine for children over the age of 5 years, while repeated exposure before the age of 5 in endemic areas has been shown. This suggests that the vaccines available miss a key population.

In addition, while evidence suggests that Ty21a may be cross-protective for paratyphoid, the ViCPS vaccine targets an antigen that does not exist in Paratyphi and even some strains of Typhi (51, 54). Further, because the oral vaccine is a live, attenuated *Salmonella* strain, it is not suitable for use in immune-compromised patients, posing a challenge to widespread use in areas co-endemic for HIV. Thus, currently available vaccination strategies are not adequate to allow control of systemic typhoidal disease (53). Whether currently available vaccines mediate any protection to non-typhoidal systemic diseases has not been thoroughly characterized. These data emphasize a need to better understand the immune response during systemic *Salmonella* infections, to inform better vaccine design.

IMPORTANCE OF Th1 CELLS AND IFN-γ IN INTRACELLULAR INFECTIONS

As mentioned earlier, some bacteria and other pathogens have developed the capacity to reside within cells and effectively hide from extracellular immune recognition. Often, these pathogens enter the cells initially using the cells' own phagocytic capacity, but then are able to escape phagolysosomal degradation or escape the phagosome entirely, by a wide range of different mechanisms (49, 55). Given this unique lifestyle, intracellular pathogens require a special type of immune response designed to recognize infected phagocytes and mediate either killing of the infected cell or internal pathogen killing mechanisms. CD8 T cells have cell-specific cytotoxic capacity, allowing directed killing of infected cells, while both CD8 and CD4 T cells can secrete pro-inflammatory cytokines that activate phagocytes to initiate internal mechanisms of pathogen destruction.

CD8 T cells have multiple cytolytic capacities initiated by TCR interactions, including release of secretory granules and death receptor-mediated apoptosis. However, while these responses have a critical role in anti-viral defenses, their role against other intracellular pathogens is limited (56). Of more importance to intracellular bacterial infections, CD8 can produce pro-inflammatory cytokines such as IFN-γ, which activates macrophages to undergo changes that alter the intracellular environment to become less hospitable to the invading pathogen. While CD8 T cells respond to MHC-I complexes that mostly present antigen processed from the cytosol of nearly all cell types, CD4 T cells respond to MHC-II presented antigen on special APCs derived from the endocytic pathway. This allows CD4 T cells to recognize antigens from pathogens hiding inside of cells within endosomes, as well as antigens taken up from outside of the cell (57). Reliance on different antigen processing pathways and consequent MHC presentation allows a partial division of labor between the CD8 and CD4 T cells, although they have retained some redundancy in critical functions, such as production of IFN-γ.

Among the various CD4 T cell subsets, the CD4 Th1 cells provide the primary response to intracellular pathogens. As mentioned earlier, after CD4 T cells are activated they receive a differentiation signal that determines their cytokine profile. In Th1 cells, IL-12 upregulates the transcription factor T-bet, which is required by CD4 T cells for IFN-γ production (58). Once activated, Th1 cells are programed to secrete pro-inflammatory cytokines that include IFN-γ, TNFα, and IL-2 upon re-stimulation. In contrast, CD8 T cell priming is not usually thought of in terms of differentiation of particular subsets with distinct functions, although functional subsets have been described. Termed Tc1, Tc2, and Tc17 in reference to their Th counterparts, these cells are found in relatively low-frequency under normal circumstances, and the mechanisms driving the development of these alternative CD8 T cells remain poorly understood (58). Signal 3 was initially identified in CD4 and CD8 T cells simply as the inflammatory cytokine(s) required to induce proliferation and differentiation to effector capacity (59).

Type I interferons and IL-12 have both extensively been shown to result in the survival, expansion, and differentiation of CD8 T cells, and are critical for the conventionally described CD8 T cell effector responses, including cytolytic activity and IFN-γ production (59–62).

Additional differences between CD4 and CD8 T cell activation include, but are not limited to a shorter required duration of antigenic stimulation in CD8 T cells (63–65), different transcriptional regulation, including partial redundancy for the transcription factors T-bet and Eomes in IFN-γ production in CD8, but not CD4 (30), and differences in cellular trafficking and antigen surveillance (66). Further, evidence suggests that CD4 T cells can help to initiate CD8 T cell priming (67, 68), and may be required for optimal CD8 memory formation, a regulatory interaction that argues against mechanistic redundancy in the activation process. Thus, while T cells have evolved to share many similar pathways, CD4 and CD8 T cells are distinct cell types with different functions and rules to govern them. When studying T cell functions it is critical to keep these differences in mind and to choose a model system capable of demonstrating the full potential of the cell of interest. Accordingly, the strong requirement for Th1 cell functions in *Salmonella* infections makes this model system ideal for study of Th1 responses (9–11).

The cytokine IFN-γ is especially important during intracellular infection because of its critical capacity to activate macrophages to become M1, or classically activated, macrophages. M1 macrophages modify their internal environment to become as inhospitable as possible, including production of anti-microbial compounds like reactive oxygen and nitrogen species, as well as themselves secreting pro-inflammatory cytokines (69). The importance of IFN-γ-mediated macrophage activation is highlighted by the effects, resulting from the loss of IFN-γ or IFN-γ-inducing cytokines and transcription factors (11, 70, 71). In both mice and human beings, loss of IFN-γ results in an inability to effectively clear intracellular pathogens (72). Further, in human beings with chronic granulomatous disease or mycobacterial granulomas, IFN-γ is an effective, albeit toxic, therapeutic (73–75). Combined, these studies clearly demonstrate the requirement of IFN-γ for effective clearance of intracellular pathogens.

PLAYING HARD TO GET: HOW *SALMONELLA* SUBVERTS THE Th1 CELL RESPONSE

The critical role of Th1 cells in *Salmonella* clearance makes them an obvious target for immune evasion strategies. While there is extensive information available on the ways that *Salmonella* has found to manipulate the system (76, 77), we focus here particularly on immune evasion strategies that impair the ability for T cells to recognize their specific antigen. Three of the ways that *Salmonella* have developed to achieve this include downregulation of antigens that may be recognized by T cells, effects on TCR expression or function, and impairment of MHC processing or presentation of peptides (**Figure 4A**). The active avoidance of TCR recognition employed by *Salmonella* provides one possible explanation for the maintenance of a TCR-independent T cell stimulatory pathway.

When *Salmonella* change environments, from contaminated source to intestine to myeloid cells, their expression of antigens recognized by T cells rapidly changes (8). In addition to responding to a change in needs, this antigenic shift acts as a highly effective immune evasion strategy, resulting in the activation and expansion of large numbers of T cells that specifically recognize antigens that will not be present at the site of infection. The T cell response to FliC was shown to be inefficient at resolving infection over a decade ago, and both SipC and FliC elicit an early T cell response despite rapid downregulation of these antigens by *Salmonella* (7, 78, 79). Thus, much of the early T cell response may develop to antigens that are not available at the site of infection, preventing cognate activation for these cells.

Additionally, a number of mechanisms have been demonstrated by which *Salmonella* causes downregulation of the TCR on T cells (80). Flagellin stimulation has been shown to result in upregulation of SOCS1, which impairs TCR expression in T cells (81). Further, direct contact of *Salmonella* with T cells results in secretion of the enzyme L-asparaginase II by *Salmonella*, which breaks down L-asparagine and consequently impairs T cell blastogenesis, proliferation, and cytokine secretion by downregulating the TCR β chain (82, 83). While these mechanisms may impair the initial priming of T cells, the requirement for direct contact between T cells and bacteria in some of these studies suggests that the importance of these evasion techniques at sites of infection.

Finally, many strategies have been demonstrated by which *Salmonella* is able to inhibit either the processing of antigens into peptides or the presentation of these antigenic peptides on the surface of APCs within the MHCs (80, 84). Nearly 20 years ago, the two-component regulatory system member PhoP was shown to impair processing and presentation of antigens in macrophages (85). Further, numerous *Salmonella* pathogenicity island-2 (SPI-2) effector proteins have been implicated in impaired MHC function, including impaired loading of peptides onto MHC, prevention of lysosomal degradation that results in decreased peptide availability, and polyubiquitination of MHC that results in degradation rather than surface expression (86–89). Each of these interactions targets a step in the antigen presentation pathway that ultimately results in an impaired ability for infected cells to signal to T cells.

Given the hindrance of cognate T cell stimulation, the ability for T cells to be stimulated by non-cognate interactions as well could play an important role in *Salmonella* clearance. *Salmonella* induces a number of different non-cognate responses via PRRs, as outlined in **Figure 4B**. These include well-characterized TLR and NLR interactions, as well as other, less well-defined PRR interactions (90). Recognition of these various non-cognate ligands results in inflammation, including production of inflammatory cytokines that can stimulate T cells. This indirect stimulation of T cells in response to non-cognate *Salmonella* products provides a complementary mechanism for T cell stimulation at sites of infection with a broad array of conserved triggers. While the multitude of Th1 evasive mechanisms accentuates the need for innate signaling pathways in the elicitation of Th1 cell functions, it is important to note that innate immune pathways are not exempt from evasion strategies (91, 92). Thus, in order to provide T cells the best chance to encounter and respond to signs of infection, redundant mechanisms for stimulation that rely on either cognate antigen or MAMP-driven inflammation have developed.

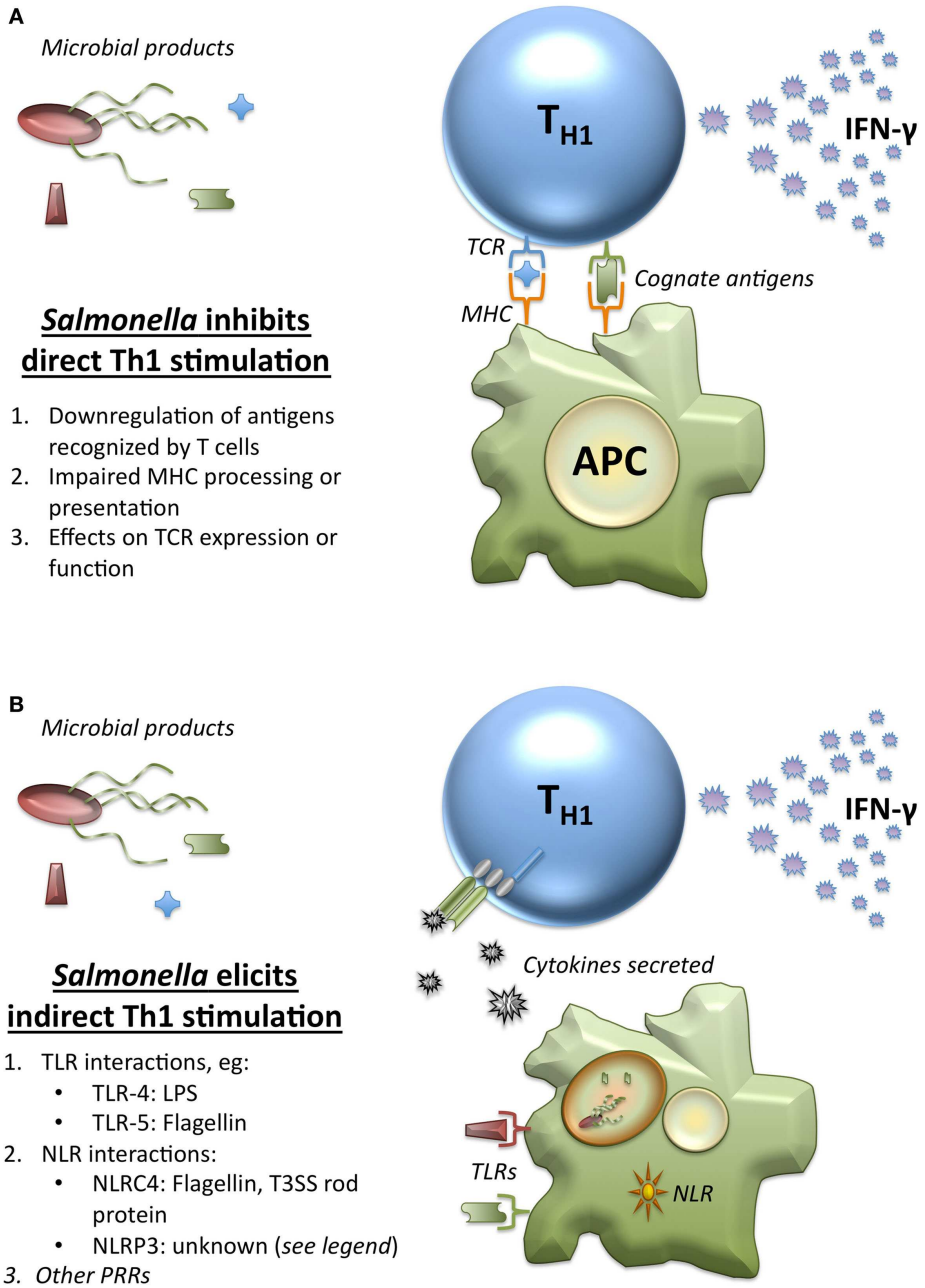

FIGURE 4 | Direct and indirect stimulation of Th1 by *Salmonella*. (A) *Salmonella* has developed multiple mechanisms to inhibit direct Th1 stimulation by cognate antigen recognition. Conventionally, *Salmonella*-specific antigens would be processed and presented by APCs on MHC-II to the TCR of Th1 cells to elicit IFN-γ at sites of infection. However, *Salmonella* has developed a wide array of strategies to limit this interaction. This includes (1) downregulation of antigens that were expressed upon entry into the host that T cells may have been primed to recognize, (2) mechanisms designed to impair processing and presentation of antigen by APCs, including downregulation of MHC itself, and (3) effects upon the expression or function of the TCR. These strategies aim to block cognate antigen recognition each step of the way, making additional non-cognate mechanisms for T cell stimulation crucial. **(B)** Many innate immune recognition pathways respond to *Salmonella*, and these elicit cytokines that allow Th1 cells to be stimulated by non-cognate pathogen products indirectly at sites of infection. (1) Several TLRs recognize conserved patterns present in *Salmonella*, including TLR-4 recognition of LPS, TLR-5 recognition of flagellin, and other TLRs that recognize bacterial nucleic acids from within the endolysosome. (2) The role of NLRs and the inflammasomes have also been well-demonstrated for *Salmonella*. While NLRC4 is known to recognize both flagellin and T3SS (Type 3 secretion system) rod proteins, the exact ligand recognized by NLRP3 from *Salmonella* is unknown. NLRP3 recognizes a number of nuclear components, which act as danger signals when present in the cytosol, but other ligands have been proposed. (3) Although less well-characterized, other classes of PRRs have the potential to recognize *Salmonella*, including the various cytosolic nucleic acid sensing receptors.

DISCUSSION

Herein, we have discussed a number of advantages to studying Th1 cell responses in a *Salmonella* model. The strong understanding of both CD4 T cell and innate immune pathways during the response to *Salmonella* infection provides a strong foundation to further explore the potential interaction between these pathways. Moreover, the tools for these studies are readily available in such a well-studied and easily manipulated model pathogen. Together, these factors make *Salmonella* an ideal model pathogen for the study of non-cognate Th1 cell responses.

Furthermore, we highlight a number of questions that remain to be answered in the study of non-cognate T cell interactions. One such question: what is the fate of Th1 cells after non-cognate stimulation? While it is possible that cells undergo the same response and fate following either cognate or non-cognate secondary interactions, there is still limited understanding of the effect of multiple stimulatory interactions with T cells. It is possible that the heightened effector response that occurs after re-stimulation impacts the fate decisions of effector T cells. It is also possible that the combination of cognate activation and non-cognate stimulation could regulate cell fate differently than solely cognate interactions. If that is the case, non-cognate stimulation could result in apoptosis and the subsequent loss of these cells, or it could act as a signal for a cell that should transition to memory during contraction. Within the memory pool, there may be a subtle difference between cells that did or did not receive non-cognate stimulation during their effector phase. Further work is required to determine what the impact of this response pathway is on T cell fate decisions.

Additionally, more work is necessary to clarify the requirements and role of non-cognate interactions of memory CD4 T cells. Our data have shown that the response of memory T cells to LPS alone after *Salmonella* clearance is much lower than the response observed during infection (35). However, it is not yet clear why so few memory T cells were able to respond. Could they require cognate re-activation first, and if so, why? Does the active infection contribute something necessary for non-cognate T cell stimulation, such as inflammasome activation or an additional cytokine? If something is missing, why are any memory T cells able to respond to the stimulation? Is there something different about these memory cells that retain innate stimulatory capacity? Understanding the memory response is critical to understanding the role of non-cognate stimulation, in particular, when trying to apply these findings to the improvement of vaccine strategies or to understanding a possible role in autoimmune disease.

Finally, while much of the early work in describing cognate T cell interactions was done *in vitro*, recent advances in the capacity of live *in vivo* imaging technologies have allowed for real-time observations of these complex interactions. This system is advantageous because it allows for individual cell tracking and a chronological history of a specific cell under natural conditions. For example, during the activation process an individual T cell may interact with many separate DCs, and these interactions have a cumulative effect upon T cell function (63). Further, it allows exploration of such brief conversations as occur, for instance, at the kinapse, which are otherwise difficult to capture due to their transience (93). Unfortunately, there are currently technical limitations that could make visualizing T cell responses to *Salmonella* difficult. As the technology continues to improve, studies such as these will open the door for a new understanding of the dynamic complexity of T cell interactions within the contexts of time and space that have, until now, proven particularly challenging for immunologists (94). Similarly, it may soon be possible to watch cognate and non-cognate T cell interactions as they occur to begin to answer questions about non-cognate Th1 stimulation such as those posed here.

As the mechanisms underlying non-cognate Th1 stimulation become clearer it may also become possible to define the relative contributions of cognate and non-cognate T cell interactions at various stages in the Th1 response. For example, in Section "Bystander Activation and Non-Cognate Stimulation," we discuss recent evidence that Th1 cells require IL-18 receptor signaling in order to respond to non-cognate signals but not for initial TCR-dependent activation. These differential pathways provide one possible tool for separating cognate and non-cognate response roles. To deeply explore these responses and their outcomes, a system in which each pathway could be selectively and transiently inhibited would be ideal. Such studies could be critical to understanding the role of infection and inflammation in T cell responses.

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Antibodies and protection against invasive *Salmonella* disease

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Invasive disease caused by *Salmonella enterica* is a major global public health concern. It has two main clinical forms: enteric fever and invasive nontyphoidal *Salmonella* (iNTS) disease. Enteric fever imposes its highest burden of disease in South and South-East Asia and is principally caused by *S.* Typhi and *S.* Paratyphi A. Conversely, iNTS disease is a particular problem in sub-Saharan Africa where it is a leading cause of bacteremia (1, 2) and is mainly caused by *S.* Typhimurium and *S.* Enteritidis. As facultative intracellular bacteria, *Salmonellae* persist and multiply within the intracellular niche in macrophages but they are also capable of independent cell-free existence and this enables the spread of the infection from macrophage to macrophage. The way the immune system protects against these two phases of infection differs and this is key for developing a strategy to induce protective immunity against *Salmonella*. Antibodies have an important role in eliminating extracellular bacteria, while specific T cells are important for the clearance of intracellular bacteria. The contribution of these two arms of acquired immunity against *Salmonella* infections to protection has been an area of controversy in the past, and their relative importance is only now emerging. This opinion piece focuses on the role of antibodies in protecting against invasive *Salmonella* disease, and the application of this to vaccine development.

Epidemiological investigation, *in vitro* studies, animal models and vaccine studies indicate that antibodies can kill *Salmonella* that are not shielded by residing inside host cells. Conceptually, *Salmonellae* are exposed and therefore vulnerable to antibodies at distinct points of the invasion cycle: following initial invasion, when first entering the circulation, and when transiting from one phagocyte to another via the blood or extracellular fluids (3). An important consideration is the time that these bacteria are exposed to antibodies and whether this is sufficient for antibody-induced killing to occur. *In vitro* kinetic studies indicate that there is a window of opportunity of approximately 10 min before extracellular *S.* Typhimurium are killed by antibody and complement, and this time is sufficient to allow a proportion of bacteria entering the blood to escape into the intracellular niche (4).

Candidate vaccine studies in mice, where immunization is followed by challenge with live *Salmonella*, indicate the importance of antibodies for protection. Several studies have investigated experimental conjugate vaccines based on purified O polysaccharide from *Salmonella* (O-antigen; O:4,5 for *S.* Typhimurium and O:9 for *S.* Enteritidis) (5). Unlike intact lipopolysaccharides, these O-antigens lack lipid A and so are unable to act as thymus-independent type 1 (TI-1) antigens. Because of their repeating structure, they are likely to behave as thymus-independent type 2 (TI-2) antigens (6, 7) and therefore be capable of inducing *Salmonella*-specific antibodies, but not T cells. If O-antigen is associated with *Salmonella* protein, as it is in the intact bacterium or when present in membrane-vesicle-preparations, it has the potential to induce T-dependent B-cell immunity. T-cell help permits an immune response to the O-antigen in infants, affinity maturation of the antibody response and results in more persistent antibody production and the induction of memory.

Passive transfer studies of antibody from immune to non-immune animals have confirmed an important role for antibodies in protecting against *Salmonella* in mice. Nevertheless, the protection that antibody confers in this model depends on the inherent resistance to *Salmonella* of the mouse strain used, the virulence of the *Salmonella* strain, and the design of the challenge study. Optimal protection against *Salmonella* in mice requires a combination of antibodies and T cells. T cells appear to be most important for the late clearance of *Salmonella* infection (8), involving killing of intracellular bacteria from the macrophage beds of the spleen and liver.

There are a several drawbacks to studying *Salmonella* infections in mice as a model of disease in humans. These include the human restriction of *S.* Typhi and Paratyphi A, which limits mouse studies to nontyphoidal serovars. Also, there are differences in antibody-mediated immunity to *Salmonella* in mice and men. In man, antibodies can kill through direct complement-fixation and opsonophagocytosis, while in mice there appears to be little complement-mediated killing (9), leaving opsonophagocytic mechanisms to effect killing. In man, although there is evidence regarding the mechanisms of immune protection from vaccines against typhoid fever, no vaccine against NTS has progressed beyond a phase I clinical study. Hence, inferences regarding the mechanisms of

immunity to iNTS disease in man come primarily from immunoepidemiological studies.

Of the two widely used types of vaccine against typhoid, Vi capsular polysaccharide (Vi CPS) vaccine, appears to operate entirely through the induction of protective antibody (5, 10). Similar to pure O-antigen, Vi polysaccharide is likely to be a TI-2 antigen. Despite lack of conjugation to a protein moiety, and hence lack of induction of T-cell immunity, the antibodies induced confer 55% 3-year protection (10, 11). New vaccines, where Vi CPS is conjugated to carrier proteins, such as tetanus toxoid, have been licensed recently for in-country use in India and China. These vaccines should provide greater protection than their unconjugated predecessor, albeit through more persistent and higher affinity Vi antibody production, rather than eliciting *Salmonella*-specific T cells, as the carrier proteins are usually not *Salmonella*-derived. The other widely used vaccine against typhoid is Ty21a, a live attenuated vaccine capable of inducing T cells as well as antibodies against *Salmonella*. Ty21a has a similar reported (51%) 3-year efficacy against typhoid as Vi CPS vaccine. Although Ty21a induces antibodies, none are directed against Vi, since it lacks Vi expression. The mechanisms by which Ty21a confers its protection are not well understood, but may include antibodies against the O:9 antigen which *S.* Typhi shares with *S.* Enteritidis.

The absence of a licensed vaccine against NTS means there is neither vaccine efficacy data nor a correlate of protection for iNTS disease. Consequently, evidence of the importance of antibodies in protection against iNTS disease relies on epidemiological evidence, which shows a correlation between fatal systemic iNTS disease and the period in childhood when naturally acquired antibodies are absent. This occurs after maternally-transmitted antibody has waned and before antibody has been induced through exposure, with peak incidence around one year of age (12). Although typhoid fever and iNTS disease are caused by bacteria belonging to the same species, extrapolating mechanisms of protection from the one disease to the other is not straightforward. One reason for this is the Vi capsule of *S.* Typhi which is absent from almost all nontyphoidal serovars of *Salmonella*. The capsule has immunomodulatory effects and has been shown to reduce inflammation (13–15). Additionally, it is now clear through genotyping that although the *S.* Typhimurium in sub-Saharan Africa is serologically indistinguishable from strains in the US and Europe, they belong to a different clade (16, 17).

Typhoid fever and iNTS disease have very different clinical manifestations and may require different approaches in order to effect protection by vaccination. Differences in their associated comorbidities, in particular, imply that the mechanisms of immune protection against these two forms of invasive *Salmonella* disease are not the same. HIV-infected individuals are highly susceptible to iNTS disease, while this association is not present with typhoid fever. Epidemiological data from Tanzania suggest a protective effect of HIV infection against typhoid, while an association between malaria and iNTS disease has long been recognized. Once again, no such link appears to exist with typhoid. Finally, individuals with deficiencies of the IL12/23-IFNγ cytokine axis (T_H1 deficiencies) commonly present with iNTS disease, but not typhoid fever.

As discussed above, the acquisition of antibodies against NTS with age among African children corresponds to a fall in the incidence of episodes of iNTS disease (12), thus supporting a role for antibodies in protection against iNTS disease among young children. These antibodies have been shown to induce killing of *Salmonella* by phagocytes (18) and complement alone (12). More recently, this early acquisition of antibody-mediated immunity has been shown to correlate with levels of antibodies to O-antigen (19), supporting the development of a vaccine that induces such antibodies in order to protect young children in Africa against iNTS disease. Surprisingly, acquisition of *Salmonella*-specific T cells coincides with a peak in age-related iNTS disease incidence in African children (19), but these T cells could still play a secondary role in immunity to *Salmonella* among such children. Hence, in otherwise immune-competent children, a vaccine that can induce antibodies, particularly antibodies to O-antigen, appears likely to protect against iNTS disease. The early acquisition of antibodies to O-antigen occurs even in locations such as USA (20), where iNTS disease is uncommon, suggesting either ubiquitous sub-clinical exposure to NTS or the development of cross-reactive antibodies from other immune stimuli.

The reasons why HIV-infected individuals are susceptible to iNTS are more complex. While those with CD4 counts below 200/μl are most susceptible, the relevant mechanisms are probably more than just a reduction in $CD4^+$ T cell-afforded protection. Dysregulation of anti-iNTS antibody-specific antibody production and cytokine responses, and increased invasion of *Salmonella* across the gastrointestinal wall also seem to be contributory, as does $CD17^+$ T cell deficiency (2). Levels of antibodies against *Salmonella* O-antigen are much higher in some HIV-infected, compared with HIV-uninfected individuals and are associated with a lack of complement-mediated killing of *Salmonella in vitro* (21). Although the clinical significance of these findings is not entirely clear, we have recently described a similar occurrence in a group of patients with bronchiectasis and chronic *Pseudomonas aeruginosa* lung infection. High levels of IgG2 antibodies to the O-antigen of *Pseudomonas* are associated with both impaired *in vitro* killing of these bacteria, increased severity of respiratory infections and poor lung function (22). Nevertheless, anti-O-antigen antibodies are bactericidal at lower concentrations (23). Recurrent episodes of iNTS disease are a common problem among HIV-infected African adults. Many are caused by the same isolate of NTS suggesting persistence of *Salmonella* infection (24), even after clinical remission. With likely persistence in the intracellular niche and the importance of T cells for clearance of intracellular infection, a vaccine capable of inducing *Salmonella*-specific T cells may be more important in the context of HIV infection than the immune naivety of infancy.

Perhaps surprisingly, iNTS disease is not a common feature in individuals with primary antibody deficiencies, such as X-linked agammaglobulinemia or common variable immunodeficiency, although *Salmonella* gastrointestinal disease has been reported to be a problem in

cohort studies of such patients (25). If, as other evidence indicates, antibody is key for protection against *Salmonella*, a lack of iNTS disease may be due to the low prevalence of *Salmonella* infections and absence of the ST313 invasive *S.* Typhimurium pathovar in industrialized settings, where it is possible to make a diagnosis of antibody deficiency. Alternatively, there may be redundancy in immunity to *Salmonella* by the time antibody levels wane and patients with CVID present with recurrent infections.

On the other hand, the high incidence of NTS granulomata among individuals with IL12/23-IFNγ cytokine axis deficiencies shows that antibodies (and complement) are insufficient alone to protect against iNTS disease in man. One series reported *Salmonella* disease in 43% of individuals with IL12/23 or IL12/23-receptor deficiencies (26). This cytokine axis is important for macrophage activation and elimination of intracellular bacteria, in particular *Salmonella* and mycobacteria. When tested, these patients have antibodies against *Salmonella*. Since they do not succumb to their *Salmonella* infections, it is plausible that, while not being sufficient to clear the macrophage beds of intracellular *Salmonella*, antibodies prevent fatal disease. As with HIV infection, the *Salmonella* serovars isolated are almost always nontyphoidal. Since IL12/23-IFNγ cytokine axis deficiencies affect signaling in response to *Salmonella* by innate/innate-like lymphocytes, including NK cells and γδ-T cells (27), the occurrence of *Salmonella* disease does not necessarily imply the need for *Salmonella*-specific T cells. iNTS disease is common among individuals with chronic granulomatous disease (25) and African children with malaria (28). In both cases, the phagocyte oxidative burst mechanism is impaired, suggesting the requirement for a functioning innate immune system to protect against iNTS disease.

In conclusion, vaccine efficacy studies strongly support a role for antibodies in protection against typhoid fever. Immunoepidemiological studies from Africa also support the importance of antibody for protection against fatal iNTS disease. However, strong clinical associations with secondary immunodeficiency due to HIV infection and malaria, and primary immunodeficiencies of the IL12/23-IFNγ cytokine axis and chronic granulomatous disease, suggest that antibody-mediated immunity against iNTS disease is only fully effective in the presence of an otherwise intact immune system. These observations indicate that bacteria from the same species (*Salmonella enterica*) not only cause different diseases, but that different immune mechanisms protect against these diseases. This conclusion may be applicable to other bacterial pathogens. While an antibody-inducing vaccine against iNTS disease may protect immunologically naive, but otherwise immunocompetent young children, it may be insufficient to protect individuals with primary and secondary immune deficiencies. Ultimately, efficacy studies with vaccines against NTS will be required to understand the importance of antibodies against iNTS disease. With no such vaccine currently even in early stage clinical trials, we are set for a long wait.

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Salmonella modulates B cell biology to evade CD8$^+$ T cell-mediated immune responses

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Although B cells and antibodies are the central effectors of humoral immunity, B cells can also produce and secrete cytokines and present antigen to helper T cells. The uptake of antigen is mainly mediated by endocytosis; thus, antigens are often presented by MHC-II molecules. However, it is unclear if B cells can present these same antigens via MHC-I molecules. Recently, *Salmonella* bacteria were found to infect B cells, allowing possible antigen cross-processing that could generate bacterial peptides for antigen presentation via MHC-I molecules. Here, we will discuss available knowledge regarding *Salmonella* antigen presentation by infected B cell MHC-I molecules and subsequent inhibitory effects on CD8$^+$ T cells for bacterial evasion of cell-mediated immunity.

Keywords: B cells, *Salmonella*, CD8 T cells, cross-presentation, PD-L1

INTRODUCTION

Salmonella typhi is the causative agent of typhoid fever in human beings, while infection with *Salmonella enterica* serovar Typhimurium (*Salmonella typhimurium*) produces a systemic illness in mice similar to that in human beings (1). In susceptible mice, the bacteria reside inside *Salmonella*-containing vacuoles (SCVs) of neutrophils, macrophages, and dendritic cells, in which they replicate, resist killing, and induce systemic disease (2–5). Uptake of *Salmonella* is mediated by the coordinated action of several virulence proteins translocated through the type III secretion system (T3SS), encoded by genes of *Salmonella* pathogenicity islands (SPIs) (6). While SPI-1 genes encode T3SS translocated proteins essential during bacterial invasion, T3SS SPI-2 genes are expressed once the bacteria are within the phagosome (7).

The bacteria exploit several types of immune cells for long-term survival (8–10). To survive within these cells and promote colonization, the bacteria release several virulence proteins that alter host cell functions, such as cytoskeletal architecture, membrane trafficking, signal transduction, cell death, cell trafficking, and cytokine gene expression (5, 6). This review focuses on the role of B cells during *Salmonella* infection, specifically as a niche from which the bacteria can evade immune responses and survive long-term within the host.

GENERAL ANTIGEN PROCESSING AND PRESENTATION

Antigen location influences its proteolytic processing pathway and its access to different classes of MHC molecules. Subsequent presentation of these antigens by MHC-I or MHC-II molecules is necessary to induce a T cell immune response. Extracellular antigens are captured by antigen-presenting cells (APCs) through phagocytosis, macropinocytosis, or endocytosis. Newly formed phagosomes containing antigen undergo progressive trafficking characterized by acquiring or losing endosomal markers to generate a mature phagosome. Finally, their fusion with lysosomes allows complete degradation of their cargo due mainly to serine proteases (cathepsins) (11). Assembly of peptide/MHC-II complexes takes place in a multilamellar endosomic compartment that contains newly synthesized MHC-II molecules bound with invariant chain-peptide (CLIP) and machinery necessary for efficient peptide loading. The acidic environment facilitates the exchange of CLIP for antigenic peptide, catalyzed by H-2M in mice or HLA-DM in human beings. Recycled MHC-II molecules from the cell surface can also be used to form peptide-MHC-II complexes. Then, the peptide-MHC-II complexes newly formed are transported to the plasma membrane. Finally, effective MHC-II presentation requires clustered peptide/MHC-II complexes at the APC surface that can subsequently interact with the T cell receptor (TCR) and CD4 co-receptor (11, 12).

Alternately, intracellular antigens in the majority of cells are processed within the cytosol by proteosomal degradation. The peptide fragments are then translocated to the endoplasmic reticulum (ER) lumen by the transporter associated with presentation. Nascent MHC-I molecules and β2-microglobulin associate with the ER proteins tapasin, calreticulin, and Erap57, which allows glycosylation of MHC-I and optimal folding necessary after peptide binding. Then newly peptide/MHC-I complexes are transported to the cell surface (12, 13). Stable heterotrimeric complexes are necessary to engage the TCR and CD8 co-receptor. However, extracellular antigens localized in vesicular compartments of APCs can also be efficiently presented by MHC-I molecules (14), a process known as cross-presentation or cross-priming. At least four routes for cross-priming have been described (15): (1) the cytosolic route requires peptide translocation from the phagosomes to the cytosol for their proteosomal processing and subsequent ER translocation (16); (2) the vacuolar route involves peptides generated within the phagosome be loaded in intravacuolar-recycled

MHC-I molecules (17); (3) the antigen is cross-processed through a phagosome-cytosol-phagosome alternating pathway (18); and (4) peptides are processed in a previously non-characterized endocytic compartment, secreted into the cytosol, and loaded onto empty MHC-I molecules on the surfaces of macrophages and bystander cells (19, 20).

SALMONELLA INTERFERES WITH ANTIGEN-PROCESSING MECHANISMS

Salmonella evade acquired immune responses to establish a chronic infection (21, 22). T cell responses can be inhibited by impaired APC antigen processing and presentation caused by bacterial proteins encoded by SPI-2 genes. As mentioned previously, *Salmonella* interferes with normal cell trafficking; for example, *Salmonella* protein SpiC inhibits maturation of *Salmonella*-containing phagosomes into phagolysosomes in macrophages and dendritic cells (2, 23–25). In addition, the phosphoinositide phosphatase SopB modulates vesicular trafficking (26). This virulence protein manipulates membrane surface charges of nascent SCVs by reducing levels of the negatively charged lipids phosphatidylinositol-4-5-biphosphate and phosphatidylserine, thus resulting in SCV maturation (27). Inhibition of phagosome acidification has been observed in macrophage cell lines (e.g., IC21) and may impede the proteolitic activity of cathepsins residing in late-endosomal compartments; this mechanism could also modify peptide processing prior to presentation (28). The integrity of the SCV, attributed to SifA, is also crucial for its resistance to oxidative killing mediated by the phagocyte oxidase phox (2). *Salmonella* mutants defective in SPI-1 and SPI-2 genes show reduced proliferation within macrophages, indicating these gene products could limit the source of peptides for antigen presentation, resulting in delayed T cell responses (29, 30). In support of this finding, Helaine et al. recently used fluorescent dilution to study intracellular replication of bacteria to determine the vacuolar environment induces phenotypic heterogeneity, thereby explaining the presence of non-replicating, yet persistent, *Salmonella* that could provide a reservoir for relapsing infection (31). Additionally, studies in human beings reveal the bacteria can control surface MHC-II expression through ubiquitination (32). Thus, *Salmonella* can impair antigen processing and presentation steps at multiple levels to prevent activation of T cell responses.

SALMONELLA EVADE T CELL RESPONSES

An immunosuppressive effect on T cells, both dependent and independent of bacteria, has been observed during *Salmonella* infection. Basel Al-Ramaldi first noted this effect in macrophages infected with an attenuated strain of *Salmonella* cultured with splenocytes in transwell plates. Soluble factors mediated T cell suppression, but the exact nature of the factor(s) was not determined at that time (33). Later, T cell proliferation assays were performed in the presence or absence of the inducible nitric oxide synthase (iNOS) inhibitor L-NMMA, which showed the suppression is also mediated by dendritic cells and is dependent on NOS induction (34). When nitric oxide was blocked with aminoguanidine, the inhibition of T cell suppression, macrophage activity, and polymorphonuclear leukocyte influx was observed (35). Thus, nitric oxide may play multiple biological roles during *Salmonella* infection. Other studies employing the human-restricted strain *S. typhi* showed the polysaccharide Vi, released from *Salmonella*, leads to an impairment of IL-2 production in T cells by interacting with the membrane prohibitin complex (36). Other transwell assays with $CD8^+$ T cells and dendritic cells infected with *Salmonella* deficient in the proteins SPI-1, SPI-2, phoP, and sti or carrying virulence plasmids demonstrated priming can be inhibited by direct contact with the bacteria (37). Moreover, exposure to LPS during priming in *Salmonella*-infected mice suppressed IL-2 and TNF-α production of flagellin-specific $CD4^+$ T cells, resulting in exacerbation of murine typhoid (38).

Other mechanisms for T cell evasion *Salmonella* infection have been described. Experiments involving adoptive transfer of $CD4^+$ T cells from TCR-transgenic mice into *Salmonella*-infected mice showed the bacteria induce a progressive culling of newly activated, high-avidity, antigen-specific $CD4^+$ T cells that express higher levels of programed death-ligand 1 (PD-L1) in an SPI-2 dependent manner (39, 40). This mechanism reshapes the repertoire of antigen-specific T cells after *Salmonella* infection. Furthermore, several groups have found the bacteria are able to reach the thymus (41, 42). We have observed that infections of the thymus cause Vβ chain rearrangements of TCRs in single-positive $CD8^+$ T cells, possibly leading to a biased selection of certain types of clonal cell populations (unpublished data). *Salmonella* can downregulate TCR expression by reducing the amount of both surface and intracellular TCR-β chain in T cells co-cultured with *S. typhimurium* (43). However, it is unknown if the bacteria could trigger or produce crosstalk between signaling pathways that would lead to this phenotype.

Because regulatory T (T_{reg}) cells mediate immune suppression, these cells can play both detrimental and protective roles in host defense against infection. Johanns et al. has shown that suppressive capacity of Treg coincide with a delay of elicing protective response during early *Salmonella* infection, contrary during late infection Treg suppressive potency diminish (44). Moreover, peritoneal NK1.1 αβ T cells reduced IL-12 production in macrophages by secretion of IL-4 upon TCR activation, during the early phase of *Salmonella* infection (45). Thus, *Salmonella* employ several strategies to overcome acquired immunity in order to persist and produce a chronic infection in the host.

B CELLS AS APCs IN T CELL PRIMING

The introduction of fluorescence-activated cell sorting (FACS) revolutionized the study of B cells, allowing the classification of B cells from lymph nodes, the spleen, and more recently, from the liver (46) into phenotypically and functionally distinct populations, denoted B1 and B2. The B2 lymphocytes are further subdivided into marginal zone B (MZ-B) and follicular B (FO-B) cells, while B1 lymphocytes are grouped as B1a or B1b cells. All subsets differ in their development, location, function, and most importantly, their ability to present antigens to T cells. For B cells to become competent APCs, they first must receive signals either from the B cell receptor (BCR) or Toll-like receptors (TLRs) for activation. This feature allows enhanced B cells uptake of both soluble and particulate (phagocytosed) antigens, followed by the expression of co-stimulatory molecules and the subsequent

processing and presentation of antigens with MHC-I or MHC-II molecules (47).

Marginal zone B cells are strategically located in the bloodstream for easy activation and to intercept and react to blood-borne antigens (48, 49). Antigens captured by MZ-B cells are delivered to follicular dendritic cells through shuttling dependent on the CXCR5-$S1P_1$-$S1P_3$ axis (50). However, MZ-B cells can also initiate a rapid first line of defense, demonstrated by Olivier et al., in which they express higher basal levels of co-stimulatory molecules CD80 and CD86, which are rapidly upregulated within 6–24 h after LPS exposure or BCR signaling. In fact, LPS-stimulated MZ-B cells induced a vigorous proliferation of alloreactive T cells *in vitro*, in contrast with LPS-stimulated FO-B cells, which then developed into mature plasma cells (51). In another set of experiments, Attanavanich et al. demonstrated that *in vivo* hen egg lysozyme (HEL)-specific MZ-B cells are more potent activators of naïve TCR-transgenic $CD4^+$ T cells than HEL-specific FO-B cells. The MZ-B cells likely have better access to the antigen and can rapidly migrate toward the T cell area, followed by plasma cell differentiation (52). Together, these experiments highlight the role of MZ-B cells to provide a bridge between innate and adaptive immune responses.

Similarly, B1 cells express higher basal levels of CD80 and CD86, suggesting their potential role in rapidly initiating a T cell response (53). The capability of peritoneal cavity B1 cells to phagocytose, process, and present particulate antigens, such as OVA bound to latex beads (1 μM) (54). Interestingly, MZ-B cells, in conjunction with B1 cells from either the spleen or peritoneal cavity, participate in the response against blood-borne antigens (55). In addition to MZ-B and B1 cells, parabiosis studies suggest that mature B cells located in the perisinusoidal niche of bone marrow, which have access to the circulatory system and can freely enter and exit the bone marrow, are also specialized for T cell-independent responses to blood-borne antigens (56). Previous paradigms describing B cell antigen presentation have changed, further supported by recent findings involving phagocytic IgM^+ cells from teleost fish and amphibians that indicate an evolutionary relationship between B cells and macrophages (57). This theory suggests B cells may have evolved from ancient phagocytic cells to macrophage-like cells to B cells that maintained their ability to phagocytose. Therefore, when B cells are activated, they become potent APCs when they encounter specific antigens, leading to cognate T-B cell interactions, T cell activation, and germinal center (GC) reactions. The amount of antigen captured and presented by GC B cells to follicular helper T (T_{fh}) cells is proportional to cell division and hypermutation rates because GC B cells with the highest affinity for antigens are selectively expanded and diversified (58).

In addition to priming T cells, APCs can also provide signals that instruct T cells to enter into effector/memory differentiation programs. Soo Choi et al. found that T_{fh} differentiation is mediated by two key players; during priming, dendritic cells induce Bcl6 expression in T_{fh} cells, while the stable commitment to this differentiation program requires interaction with FO-B cells (59). This mechanism was explored by experiments in which antigen-specific T cells from MD4/μMT B cell-deficient mice showed reduced levels of Bcl6 expression at day 7 post-immunization against lymphocytic choriomeningitis virus (LCMV). Experiments using B cell/dendritic cell MHC-II-deficient mice reinforced the role of MHC-II in antigen presentation by FO-B cells in cooperation with T_{fh} differentiation (60). This model suggested B cells participate in the initiation, maintenance, and full polarization of T_{fh} differentiation (61). Regarding T_h1 differentiation, Barr et al. have shown that an antigen-specific IgG2c primary response is absolutely dependent on MyD88 signaling to B cells in mice immunized with T cell-dependent antigen or in mice infected with *Salmonella* (62). They also found that B cell-intrinsic MyD88 signaling is required for primary effector T_h1 cell development, whereas antigen-specific BCR-mediated presentation is necessary for the development of T_h1 memory cells against *Salmonella* (63). In addition, MZ-B cells participate in T_h1 cell differentiation, and Attanavanich et al. found that, when cultured *in vivo*, HEL-primed MZ-B cells from MD4 mice with naïve $CD4^+$ T cells produce large amounts of T_h1-like cytokines and IFN-γ but low levels of IL-4, IL-5, and IL-10. This expression pattern suggests MZ-B cells also provide signals for T_h1 cell development during the primary immune response (52). These findings emphasize the non-redundant role of B cells as programmers of $CD4^+$ T cell differentiation.

The ability of B cells to process and present viral antigens to $CD8^+$ T cells via MHC-I molecules was first explored by Ciavarra et al. with proliferation and cytotoxicity assays using $[^3H]$thymidine and ^{51}Cr release, respectively. These experiments highlighted the efficacy of mitogen (LPS)-activated B cells in displaying target antigens on their cell surface membranes, which are efficiently recognized in a MHC-I-dependent manner by vesicular stomatitis virus-specific cytotoxic T cells (CTLs) (64). Other experiments employing mice infected with LCMV-Clone 13, a strain that causes persistent infections, showed that neutralizing antibodies are induced unless $CD8^+$ T cells were depleted. This result suggests B cells might be actively infected and capable of presenting viral peptides on MHC-I molecules; thus, they may become targets for LCMV-specific CTLs (65). Subsequent studies by the same group used ^{51}Cr release assays with splenocytes from LCMV-infected BALB/c ($H\text{-}2^d$) mice and as target, LCMV-infected, neutralizing antibody-secreting hybridomas. Showed that CTLs lysed the infected hybridomas, because LCMV was endocytosed through the membrane-anchored neutralizing antibody receptor and are later eliminated by virus-specific CTLs (66). These results reinforced the role of B-cell as APC. Although not absolutely required, they do play a role in T cell priming, thus positively impacting the function of $CD8^+$ T cells. Multiple cytokines, such as IL-2, IL-12, IL-21, IL-27, and IL-33, which are produced by $CD4^+$ T cells, APCs, and non-hematopoietic cells from the T cell zone, participate in promoting effector T cell differentiation (67, 68). Liu et al. first explored B cells' potential "helper role" for $CD8^+$ T cells by evaluating the anti-influenza cytolytic activity of $CD8^+$ T cells. They demonstrated that soluble factors released by B cells could replace the $CD4^+$ T cell requirement to induce cytotoxic responses to influenza virus (69). These previous studies changed our view of B cells as APCs and showed they strongly influence an effective $CD8^+$ T cell response against pathogens localized in the cytoplasm, such as viruses.

Evidence that B cells possess machinery to perform alternative pathways for antigen processing for $CD8^+$ T cell priming

has been presented in studies related to vaccine development and bacterial infections. For example, the *Mycobacterium tuberculosis* heat shock protein 70 (HSP70) is endocytosed, subjected to vacuolar processing, and forms highly immunogenic complexes with chaperoned peptides that are presented on MHC-I molecules to elicit a $CD8^+$ T cell response (70). In one experiment, CpG oligodeoxynucleotides-activated B cells could uptake OVA-associated HSP70, in a CD91-dependent manner, process the fusion protein by vacuolar mechanisms and prime OVA-specific $CD8^+$ T cells. In another study involving immune-stimulating complexes (ISCOMS) that induce strong MHC-I-restricted responses, HEL-specific B cells could uptake OVA-HEL-ISCOMS and then stimulate OVA-specific $CD8^+$ T cell responses. This cross-presentation required endosomal acidification, proteosomal processing, and classical MHC-I/peptide transport (71). During bacterial infections, BCR-mediated internalization of *Salmonella* led to efficient antigen delivery to MHC-II antigen-loading compartments; however, when the proteosome was inhibited with MG-132, only a partial dependence on this protease was observed (72, 73). These data indicate B cells may have machinery to employ the phagosome-cytosol antigen presentation pathway. In addition, our group has shown that *Salmonella*-infected B cells cannot use the vacuolar alternative pathway that involves antigen processing in a vacuolar compartment, which is often followed by secretion and loading of antigenic peptides to MHC-I molecules on the surface of B cells and bystander cells (28). In sum, these studies show that B cells possess machinery necessary to induce a $CD8^+$ T cell response against intracellular pathogens localized in vacuolar compartments.

We have thus far reviewed evidence that portrays B cells as highly competent APCs that positively impact T cell functions; however, B cells are also negative regulators of T cell responses therefore denoted as B_{reg} cells. Their inhibitory function has been associated mainly with IL-10 because this B cell derived-cytokine can protect against autoimmunity, yet increase the host's susceptibility to infection (74). Recently, two separate studies identified an additional soluble factor that mediates regulatory functions in B cells. One study found IL-35 can induce the conversion of typical B cells into an IL-35-producing B_{reg} cell population dependent on STAT1 and STAT3, which are induced through signaling by IL-12Rβ2 and IL-27Rα. In addition, induced B cells exerted a suppressive influence on pathogenic T_h17 and T_h1 cells from experimental autoimmune uveitis-induced mice (75). The second study revealed that B cells, through activation of TLR4 and CD40, secrete IL-35. This study focused on B35 cell-deficient mice and found B cell-derived IL-35 is necessary for pathogenic T_h17 and T_h1 cell suppression in an autoimmune encephalomyelitis model. Moreover, a lack of IL-35 production by B cells led to increased activation of macrophages and $CD4^+$ T_h1 cells and favored B cells as APCs in a *Salmonella* infection model (76).

B CELLS DURING *SALMONELLA* INFECTION

T cells, particularly T_h1 cells, are crucial for *Salmonella* infection control due to their IFN-γ secretion (62, 63, 77–81), while *Salmonella*-specific antibodies are required to resist secondary infection. The role of B cells as antibody-producing cells has been demonstrated using B cell-deficient mice (Igh-$6^{-/-}$ or Igμ$^{-/-}$) (82, 83) that were immunized with an attenuated strain of *Salmonella* and then challenged with a virulent strain; these mice could not resist the infection (84). In addition, transfer of immune serum to immunized B cell-deficient mice (Igμ$^{-/-}$) 1 day prior to challenge with virulent *Salmonella* effectively reconstituted their immunity (83). Thus, antibody-producing B cells are key players during secondary bacterial infections. Beyond this well-known role, another study suggests B cells are required for priming T cell responses during bacterial infections. Notably, Ugrinovic et al. found a reduced frequency of both IFN-γ-producing $CD4^+$ and $CD8^+$ T cells in immunized, gene-targeted, B cell-deficient Igh-$6^{-/-}$ mice. When primary B cells infected *in vitro* were cultured with *Salmonella*-specific $CD4^+$ T cells from immunized mice, they induced modest proliferation compared to *in vitro*-infected bone marrow-derived macrophages (85). More recently, Nanton et al. evaluated T cell responses against *Salmonella* using B cell-deficient JhD mice, transgenic mice with B cells that could not class switch or secrete antibodies, and mice with B cells that could not class switch but were able to secrete IgM. They observed a decrease of both IFN-γ $^+CD8^+$ or $CD4^+$ T cells, which suggested antibodies are not required for an optimal *Salmonella*-induced T_h1 response (86). Collectively, these studies show that *in vitro Salmonella*-infected B cells can moderately prime $CD4^+$ T cells and somehow participate in the activation of T cell responses. Recent findings from Barr et al. using a *Salmonella* infection model demonstrated intrinsic MyD88-derived B cell signals play a role in effector T_h1 cell differentiation (62, 63). Our group, along with others, has shown that *in vitro*-infected B cells produce IL-6 (87), which contributes to the early multistages of T_{fh} cell differentiation. Therefore, it is not surprising that B cells can also act as T cell programmers, although it is unknown if B cell infection may impair the multistage and multifactorial T_{fh} cell differentiation program.

B CELLS PRIME $CD8^+$ T CELLS RESPONSES DURING *SALMONELLA* INFECTION

Although $CD4^+$ T cells are known as key players during *Salmonella* infection, the role of $CD8^+$ T cell responses is less clear. Interestingly, $CD8^+$ T cells participate in the eradication of bacteria during secondary *Salmonella* infections, but their role in primary infection seems contradictory (80, 88, 89). Previous evidence demonstrated a null to modest participation of CTLs in mice deficient of β2m or depleted of $CD8^+$ T cells (80, 89). More recent reports focused on MHC-Ia-deficient mice (K^bD^b) demonstrated the role of $CD8^+$ T cells during the later stages of a primary infection (88). In addition, the involvement of non-polymorphic MHC-Ib (Qa-1, HLA-E, H2-M3) during the response against *Salmonella* has recently gained attention due to their role as presentation molecules for *Salmonella* antigens (90, 91).

When acting as APCs, B cells that express a *Salmonella*-specific BCR can, after bacterial internalization, reactivate human memory $CD8^+$ T cells via cross-presentation, leading to a CTL response (72). However, it is unclear if primary B cells infected with *Salmonella* by the natural entry pathway could cross-process and present *Salmonella* antigens via MHC-I. By using a *Salmonella* strain (S-OVA) that expresses the OVA peptide (OVAp) fusioned with the curli protein (Crl), we evaluate whether MHC-I present *Salmonella* antigens after infection. We found that *in vitro* and

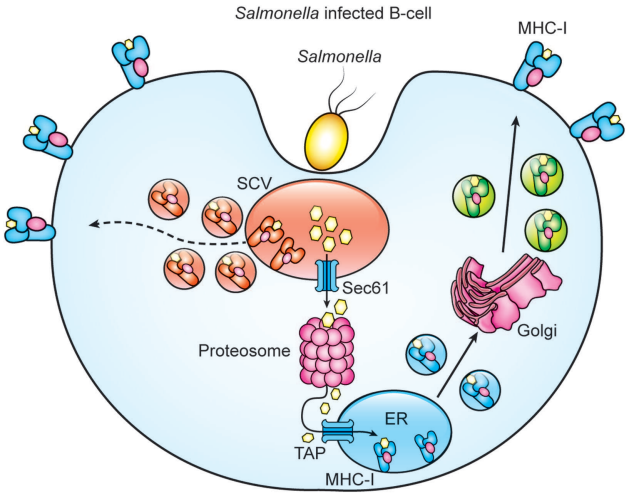

FIGURE 1 | Proteasome and paghosomal degradation are involved for cross-processing of *Salmonella* antigens by B cells. Model of cross-priming in *Salmonella*-infected B cells. *Salmonella* infection generates antigens that are translocated to the cytosol for proteosomal processing and subsequent translocation of *Salmonella* peptides by TAPs to ER for loading MHC-I. Degradation of *Salmonella* proteins with the SCV generate peptides that load recycled MHC-I molecules and the resulting MHC-I/peptide complexes are then transported to the B cell surface.

in vivo S-OVA-infected B cells express K^b-OVAp complexes. This presentation diminished by using inhibitors of the components of the classical (brefeldin and lactacystin) or vacuolar (leupeptin and ammonium chloride) pathways suggesting that processing of *Salmonella* antigens might involve the translocation of partial processed antigens from the SVC to the cytosol, followed by their proteosomal degradation and subsequent ER translocation (**Figure 1**) (unpublished data). In agreement with our results, a previous study demonstrated that, in human-specific B cells, cross-presentation of *Salmonella* antigens is partly proteosome-dependent (72). It is also likely that peptides generated within the SCV might load onto recycling MHC-I molecules (**Figure 1**). On the other hand, *Salmonella* infection also promotes B cell activation since expression of co-stimulatory molecules such as CD40, CD80, and CD86 within *Salmonella*-infected B cells is observed (unpublished data). In sum, these results suggest that infected B cells are capable of cross-processing and presenting *Salmonella* antigens and can express co-stimulatory molecules to become professional APCs that prime and sustain a $CD8^+$ T cell response. Similarly, other studies employing antigen-specific B cells in *Salmonella* infection or diabetes type-1 models further support the capability of B cells to process and present exogenous antigens (72, 92).

B CELLS AS TROJAN HORSES DURING *SALMONELLA* INFECTION

We and other groups have demonstrated that *S. typhimurium* infects and persists long-term in splenic and lymph node macrophages, splenic dendritic cells, splenic B cells, bone marrow B cell precursors, and plasma cells. Studies in human beings have shown that *S. typhi* can be isolated from bone marrow cultures, regardless of disease stage or type of pharmacological treatment (93). Bone marrow B cells present a safe niche for *Salmonella* because they cannot enter peripheral circulation until they fully mature. In human beings and mice, most *Salmonella* infections occur in the ileum, spleen, and liver (94). More recently, gallstone biofilms and the gallbladder epithelium were demonstrated niches for chronic *Salmonella* infections; however, only 3–5% of *S. typhi*-infected individuals develop a chronic infection in these sites (21, 95).

Macrophages often serve as host cells for *Salmonella* during acute and chronic infections, but the fate of these cells, as well as other infected cells, is unclear. In some cases, *Salmonella* induce host cell death, releasing the bacteria and disseminating the infection. Our group has shown that *Salmonella* inhibit pyroptosis in B cells because they abrogate IL-1β production by impairing NLRC4 transcription; thus, B cell death is not induced (87, 96). This mechanism could allow *Salmonella* survival within these cells during an innate immune response. Using the B cell line A20, we discovered the vacuolar compartment in which *Salmonella* reside is different from that in macrophages (28). Interestingly, fluorescent dilution analysis revealed that the SCV environment and nutritional deprivation of infected macrophages activate *Salmonella* virulence genes, leading to the presence of non-replicating, persistent bacteria (31). In B cells, primary infection is followed by the production of reactive oxygen species, iNOS, and pro-inflammatory cytokines IL-1β, TNF-α, and IL-6, which often control the bacteria (87). *Salmonella* replication rates within infected B cells are likely low, as we and other groups have found very few bacteria in these cells. For example, Souwer et al. used *in vitro* infection assays to determine that only 4% of human B cells phagocytose the bacteria via their BCR (73). Similarly, we have observed approximately 0.1–1% of mouse splenic primary B cells, bone marrow B cell precursors, and plasma cells get infected with *Salmonella*. However, we also discovered that after 2 months post-infection, *Salmonella* can still be isolated from infected bone marrow B cell precursors and infected plasma cells (97). Our experiments involving susceptible BALB/c mice infected with a single dose of 50 virulent *Salmonella* bacteria showed that after 1 month post-infection, bacterial CFUs could be isolated from infected splenic B1a and B1b, MZ-B, and FO-B cells (unpublished data). Thus, *Salmonella* likely exploit B cell populations to persist long-term in the host. Interestingly, if *Salmonella* infect and persist within all splenic B2 cells, infected FO-B cells, which possess migratory properties, could as act as carriers for further dissemination of *Salmonella*. Moreover, plasma cells migrate to the bone marrow and eventually undergo apoptosis (98), but these cells could also be involved in spreading the bacteria. However, it is unknown if infected splenic B cells could also differentiate into antibody-secreting cells.

The microbes' level of persistence depends on a balance between the immune response of the host and the bacteria's ability to survive within the cell. Certain viral pathogens (LCMV, HIV, HCV, HBV) (99–102), parasites (*Trypanosoma cruzi*, *Schistosoma mansoni*, *Tenia crassiceps*) (103–105), and some bacteria (*M. tuberculosis*, *Helicobacter pylori*, *Chlamydia trachomatis*) (106–108) can render T cell responses ineffective by benefiting from inhibitory stimuli such as PD-1:PD-L (PD-L1 and PD-L2) interactions. Some

of the experiments described above showed B cells can process and present *Salmonella* antigens *in vitro* and *in vivo*. In addition, we have found that B cells remain infected long-term, suggesting they may avoid elimination by CTLs. In this context, PD-L1 and PD-L2 expression in B cells infected *in vitro* and *in vivo* with *Salmonella* was observed (unpublished data). These results suggest *Salmonella* infection provides signals that trigger the transcription of PD-L1 and PD-L2 genes. Furthermore, infected B cells likely produce both positive and inhibitory signals. These inhibitory signals may be more dominant during infection because they allow the bacteria to avoid effector $CD8^+$ T cell responses. Therefore, expression of PD-L1 and PD-L2 by infected B cells could be one possible mechanism employed by the bacteria to survive within these cells and evade cell-mediated immunity. However, no current evidence indicates the PD-1:PD1-Ls axis directly terminates or attenuates $CD8^+$ T cell responses during chronic *Salmonella* infection. Our current studies simply show that *Salmonella*-infected B cells express PD1-Ls during acute and chronic infections. We have also found that PD-1 is expressed on antigen-specific $CD8^+$ T cells (unpublished data), so the participation of this axis during infection could explain why previous studies reported no significant $CD8^+$ T cell involvement during *Salmonella* infections. Furthermore, our group has also found B1 cells can produce IL-10 when infected *in vitro* with virulent *Salmonella* (unpublished data), which, along with IL-35 production, can inhibit both innate and acquired immune responses against *Salmonella* (76, 109).

CONCLUDING REMARKS

Many studies have sought to elucidate how *Salmonella* achieves a balance between avoiding immune responses and surviving long-term in its host. Previous research indicates *Salmonella* exploits several types of immune cells to persist during chronic infections. Here, we presented evidence that B cells are an amenable bacterial reservoir, promoting their persistence, dissemination, and evasion of $CD8^+$ T cell-mediated responses. Identifying the mechanisms employed by *Salmonella*-infected B cells to avoid cell-mediated immunity is clinically significant for understanding the chronic, asymptomatic carrier stage of *Salmonella* infection that occurs in human beings following typhoid fever.

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Complex adaptive immunity to enteric fevers in humans: lessons learned and the path forward

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Salmonella enterica serovar Typhi (*S.* Typhi), the causative agent of typhoid fever, and *S.* Paratyphi A and B, causative agents of paratyphoid fever, are major public health threats throughout the world. Although two licensed typhoid vaccines are currently available, they are only moderately protective and immunogenic necessitating the development of novel vaccines. A major obstacle in the development of improved typhoid, as well as paratyphoid vaccines is the lack of known immunological correlates of protection in humans. Considerable progress has been made in recent years in understanding the complex adaptive host responses against *S.* Typhi. Although the induction of *S.* Typhi-specific antibodies (including their functional properties) and memory B cells, as well as their cross-reactivity with *S.* Paratyphi A and *S.* Paratyphi B has been shown, the role of humoral immunity in protection remains undefined. Cell mediated immunity (CMI) is likely to play a dominant role in protection against enteric fever pathogens. Detailed measurements of CMI performed in volunteers immunized with attenuated strains of *S.* Typhi have shown, among others, the induction of lymphoproliferation, multifunctional type 1 cytokine production, and $CD8^+$ cytotoxic T-cell responses. In addition to systemic responses, the local microenvironment of the gut is likely to be of paramount importance in protection from these infections. In this review, we will critically assess current knowledge regarding the role of CMI and humoral immunity following natural *S.* Typhi and *S.* Paratyphi infections, experimental challenge, and immunization in humans. We will also address recent advances regarding cross-talk between the host's gut microbiota and immunization with attenuated *S.* Typhi, mechanisms of systemic immune responses, and the homing potential of *S.* Typhi-specific B- and T-cells to the gut and other tissues.

Keywords: *Salmonella* Typhi, *Salmonella* Paratyphi, enteric fever, typhoid fever, human immunity, CMI, multifunctional T-cells, microbiota

INTRODUCTION

Enteric fevers encompass typhoid fever caused by the Gram-negative intracellular bacterium *Salmonella enterica* serovar Typhi (*S.* Typhi) and paratyphoid fever caused largely by *S. enterica* serovars Paratyphi A and B (*S.* Paratyphi) (1, 2). Most cases of enteric fever are caused by *S.* Typhi (3). However, infections caused by *S.* Paratyphi A have been increasing in recent years, particularly in Asia (2, 4–7). Typhoid and paratyphoid fevers are life-threatening illnesses exhibiting very similar clinical features (2, 8). Humans are the only reservoir for these infections. The disease spreads by the fecal–oral route via contaminated food and water (9). In industrialized countries, enteric fevers are rare with most infections occurring in military personnel and in individuals traveling to endemic areas. According to the CDC, in the United States, it is estimated that ~5,700 cases of *S.* Typhi infection occur annually, mostly acquired while individuals are traveling internationally. However, *S.* Typhi and *S.* Paratyphi infections are a major public health problem in the developing world (9–13). It is estimated that 26.9 million new cases of typhoid fever occur annually with about 1% mortality (9–13). Based on data provided by the World Health Organization, 90% of these typhoid deaths occur in Asia, and most victims are children under 5 years of age (14). Furthermore, antimicrobial treatment of enteric fever and asymptomatic carriers has become increasingly complicated due to the emergence of multidrug-resistant strains of *S.* Typhi and *S.* Paratyphi A (7, 15, 16). Thus, there has been an increased emphasis on control measures, such as improved sanitation, food hygiene, and vaccination (8, 10, 17). It has also become evident that a better understanding of the host immune responses against *S.* Typhi and *S.* Paratyphi are required. This review will focus on the adaptive human immune responses [i.e., humoral and cell mediated immunity (CMI)] to *S.* Typhi and *S.* Paratyphi acquired through natural infection, experimental challenge, and vaccination. For discussions of the "mouse model of *Salmonella* infection," the reader is referred to excellent reviews included in this "Frontiers in Immunology Research Topic" compilation.

IMMUNITY ELICITED IN NATURAL INFECTIONS CAUSED BY *S.* Typhi AND *S.* Paratyphi

Salmonella Typhi is a facultative intracellular bacterium that causes an acute generalized infection of the reticuloendothelial system (RES), intestinal lymphoid tissue, and gallbladder in humans (18). Classical symptoms include gradual onset of sustained fever, chills, hepatosplenomegaly, and abdominal pain. In some cases,

patients experience rash, nausea, anorexia, diarrhea, or constipation, headache, relative bradycardia, and reduced level of consciousness (19). After *S.* Typhi ingestion, the period of incubation ranges from 3 to 21 days, with the mean incidence between 8 and 14 days (19). Without effective treatment, typhoid fever has a case-fatality rate of 10–30%. This number can be reduced to 1–4% with appropriate therapy (10). In addition, a small number of individuals become "carriers." These individuals, after recovering from acute *S.* Typhi infection, keep shedding *S.* Typhi in their feces and are able to spread the disease.

After ingestion of contaminated food or water, sufficient numbers of *S.* Typhi might survive the low pH of the stomach and cross the intestinal epithelial monolayer through mechanisms that involve M cells, dendritic cells (DC), passage through enterocytes in endocytic vacuoles, and/or disruption of tight junctions (paracellular route) (20, 21). Once in the lamina propria, *S.* Typhi can spread systemically and trigger innate and adaptive host immune responses.

Most of our knowledge of adaptive host immune responses to *S.* Typhi natural infection originates from studies involving individuals living in typhoid endemic areas (21–23). Clinical studies indicate that the development of protective immunity after recovery from typhoid fever is possible but that the frequency of individuals able to mount protective immune responses is low (22, 23). *S.* Typhi infections in individuals living in endemic areas elicit the appearance of both humoral and CMI responses. Anti-*S.* Typhi-specific antibodies against lipopolysaccharide (LPS), H (flagellin), Vi (*S.* Typhi capsular polysaccharide; virulence factor), porins, and heat-shock proteins (e.g., GroEL), among others, have been well documented in the sera of acute and convalescent typhoid fever patients (24–31). In addition, the presence of anti-*S.* Typhi secretory IgA (SIgA) was also described in intestinal fluids of typhoid patients (32). Of note, high-anti-Vi IgG antibodies are present in a considerable proportion of chronic biliary *S.* Typhi carriers, particularly in endemic areas. The presence of functional antibodies against *S.* Typhi (e.g., bactericidal activity), which increase with age has also been reported in healthy residents of typhoid endemic areas (33). However, the role that antibodies play in protection remains elusive. For example, susceptibility to typhoid infection has been reported to occur despite the presence of elevated titers of antibodies against O, H, and other *S.* Typhi antigens (22, 23, 29, 34).

Clinical observations suggest that CMI, particularly cytokines, play an important role in host defense against *Salmonella* infection. For example, increased susceptibility to invasive *Salmonella* infections, caused largely by non-typhoidal *Salmonella*, as well as a few *S.* Typhi and *S.* Paratyphi cases, have been reported in individuals with immune deficiencies for interferon (IFN)-γ, interleukin (IL)-12, IL-23, and STAT1 receptors (35–38). Moreover, significant genetic associations were reported between susceptibility or resistance to typhoid fever and HLA-DR and HLA-DQ MHC and tumor necrosis factor (TNF)-α alleles in Vietnam residents (39). Of note, although the data is sparse, it has been reported that human immunodeficiency virus (HIV) positive patients in an endemic area are at significantly increased risk for infection with *S.* Typhi and *S.* Paratyphi (40). However, these results will need further confirmation as other studies have failed to observe this association (36).

The importance of CMI in the host's response to *S.* Typhi has also been derived from early studies in acute and chronic carrier typhoid patients, which demonstrated the presence of specific CMI responses, including antigen-specific lymphoproliferation, leukocyte migration inhibition, and rosette-forming cells (32, 41–46). Moreover, elevated serum levels of IFN-γ, IL-6, and TNF-α receptor (TNF-R) p55 and TNF-R p75 were reported in *S.* Typhi and *S.* Paratyphi A-infected patients in Nepal (47). Interestingly, in these studies higher values of IL-6 and soluble TNF-R p55 were related to poorer outcome. In another study, Keuter et al. showed that levels of the anti-inflammatory mediators IL-1 receptor antagonist (IL-1RA), soluble TNF-R (p55 and p75), and IL-8 were higher in the acute phase than in the convalescent phase (48). In contrast, the production capacity of pyrogenic cytokines (TNF, IL-6) was depressed in the acute phase of typhoid fever but was restored during the convalescent phase. Of note, no differences were observed between patients with complicated or uncomplicated disease courses. These observations have been extended by recent studies in Bangladeshi typhoid patients, which have shown the induction of specific T-cell responses [e.g., production of IFN-γ, IL-17, macrophage inflammatory protein (MIP)-1β, lymphoproliferation] to purified *S.* Typhi antigens using a novel high-throughput technique (49–51). Concerning the cellular source of cytokines/chemokines, experiments using human PBMC from healthy subjects and Ty21a vaccinees have shown that, in addition to lymphocytes, stimulation with *S.* Typhi flagella induced the rapid *de novo* synthesis of TNF-α and IL-1β, followed by IL-6 and IL-10 in macrophages (52). Follow-up experiments indicated that whole-cell *S.* Typhi and *S.* Typhi flagella also have the ability to downregulate *in vitro* lymphocyte proliferation to soluble antigens and mitogens by affecting macrophage function, suggesting that *S.* Typhi components have the potential to exert both up-regulatory and down-regulatory effects on the host immune response (53). Taken together, these observations suggest that although antibodies are likely to participate in protection against typhoid fever, CMI probably represent the dominant protective immune responses that eventually lead to the elimination of these bacteria from the host.

More limited information is available regarding immunological responses in paratyphoid fever. Several reports showed the presence of serological responses against LPS and H-flagellar *S.* Paratyphi antigens using the Widal, colorimetric, and ELISA tests (7, 54). More recently, immunogenic *S.* Paratyphi A proteins expressed in bacteremic *S.* Paratyphi A-infected individuals have been identified using an immunoscreening technique (IVIAT; *in vivo*-induced antigen technology) (4). These studies identified several *S.* Paratyphi A proteins expressed *in vivo* (~20 proteins, including those involved in pathogenesis, such as fimbria, cell envelope and membrane structures, energy metabolism, and cellular proteases), which elicited antibody responses in these patients during the acute and convalescent phases. These results confirmed and extended previous studies by the same group using a different technique (SCOTS, selective capture of transcribed sequences) in Bangladeshi patients who were bacteremic with *S.* Paratyphi A and

S. Typhi (51, 55). Taken together, these observations highlight several *S.* Paratyphi A proteins, which might play an important role in *S.* Paratyphi A pathogenesis and which may serve as targets of upcoming vaccine development efforts.

Regarding CMI, as reported in typhoid fever, elevated serum levels of IFN-γ, IL-6, TNF-R p55, and TNF-R p75 were reported in *S.* Paratyphi A-infected patients (47). Moreover, a very recent manuscript described the induction of serum pro-inflammatory cytokines in Israeli travelers who became infected with *S.* Paratyphi A while visiting Nepal (6). These studies showed elevated serum levels of both pro-inflammatory and anti-inflammatory cytokines/chemokines during the acute phase, including IFN-γ, IL-6, IL-8, IL-10, IL-15, and TNF-α. Of note, no changes were observed in the serum levels of the other cytokines evaluated in these studies (i.e., IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-12p70, IL-13, IL-17, IL-23, and TNF-β). These increases in pro-inflammatory cytokines/chemokines observed in *S.* Paratyphi A infections are similar to those reported in typhoid fever, supporting the contention that similar host immune responses might be elicited in enteric fevers caused by *S.* Typhi and *S.* Paratyphi bacteria. Interestingly, elevated serum levels of pro-inflammatory (IFN-γ, IL-12, and TNF-α) cytokines but decreased levels of IL-10 were reported in patients with early non-typhoidal gastroenteric *Salmonella* bacterial clearance in stools as compared to the non-clearance group (56). It is reasonable to speculate that these observations demonstrating the increased circulating levels of both pro-inflammatory and anti-inflammatory cytokines/chemokines suggest the concomitant presence of both T effector (T_{eff}) and T regulatory (T_{reg}) responses following wild-type infection.

Another issue to consider regarding the cytokine/chemokine data in natural infections with typhoidal and non-typhoidal *Salmonella* is that although increases in circulating cytokines/chemokines are widely considered to be associated with protective responses, this might not necessarily be an accurate interpretation. In fact, it is likely that the levels of cytokines/chemokines in the microenvironments of the gut and the "RES" (e.g., regional lymph nodes, spleen, and other secondary lymphoid tissues) are not necessarily reflected in circulation. These are the sites in which most immune responses are likely to be generated, and where *Salmonella* find their niche(s) for long-term persistence, representing important sites for localized immune responses. With the information currently available, it is not possible to rule out the notion that serum/plasma levels might be a representation of a generalized pro-inflammatory response (part of the so called "cytokine storm," a surrogate marker of inflammation) in response to a systemic bacterial infection (e.g., the host's response to LPS and other bacterial antigens) rather than an effective targeted host response leading to protection.

IMMUNITY ELICITED BY EXPERIMENTAL CHALLENGE WITH WILD-TYPE *S.* Typhi (CONTROLLED HUMAN INFECTION MODEL; TYPHOID CHI)

S. Typhi is a human-restricted pathogen, i.e., there are no good animal models that faithfully recapitulate *S.* Typhi infection (57). To partially address this shortcoming, the infection of susceptible mice with *S.* Typhimurium has been used as a model for the pathogenesis of human typhoid fever (57). Although these murine models have provided considerable knowledge regarding host–pathogen interactions, they do not fully represent *S.* Typhi infection in humans (58). Furthermore, the recent availability of full genome sequences from various *S. enterica* serovars have uncovered many differences in inactivated or disrupted genes, which can explain, at least in part, the dissimilarities observed in the immune and other host responses to these enteric bacteria (58). Thus, controlled human infection (CHI, "challenge") studies in which subjects are exposed orally to wild-type *S.* Typhi, have the potential to provide a better understanding of the human immune response to infection. Additionally, these studies have the capacity to uncover the correlates of protection against *S.* Typhi, which might prove critical to accelerate the development of better and more effective vaccines to prevent typhoid and other enteric fevers (59, 60).

While challenge experiments with virulent *S.* Typhi were reported early in the twentieth century (59), University of Maryland Researcher, Dr. Theodore E. Woodward, is considered the pioneer in the establishment of a reproducible challenge model (61). In this challenge model, participants were orally challenged with wild-type *S.* Typhi suspended in milk, without buffer. In his first challenge assay performed in the 1950s, Dr. Woodward used the wild-type strain Ty2 isolated from an outbreak in Kherson (in modern day Ukraine) in 1918 (62). All subsequent challenge assays were performed using the Quailes strain, which was isolated from the gallbladder of a chronic carrier, and demonstrated virulence through transmission to several household members (60). To highlight the importance of this challenge model, studies by Dr. Woodward and his collaborators at the University of Maryland led to the successful use of chloramphenicol in the treatment of patients with typhoid fever (61) and also served as the first step toward eventual licensure of the Ty21a typhoid vaccine (63).

Very recently, over three decades after the last human wild-type *S.* Typhi challenge study was performed at University of Maryland, Dr. Pollard's group in Oxford (UK) has re-established this model. This CHI model followed in the steps of previous studies by challenging healthy adult subjects with wild-type *S.* Typhi Quailes strain (63). However, the challenge agent was suspended in a sodium bicarbonate solution rather than milk. Two dose levels (10^3 or 10^4 colony-forming units) resulted in attack rates of 55 or 65%, respectively. Interestingly, participants who developed typhoid infection demonstrated serological responses to flagellin and LPS antigens by day 14, while no changes were observed in the titers of these antibodies in participants not succumbing to infection after challenge. It is reasonable to speculate that the increased anti-LPS responses in subjects who developed typhoid was largely the result of clinical disease involving local and systemic infection rather than representing a protective mechanism at play. Moreover, anti-*S.* Typhi antibody baseline titers did not correlate with subsequent infection risk (63). These results are somewhat different than those from Maryland challenges in which anti-H antibodies appear to correlate with protection. Of note, in the Oxford CHI studies, antibody responses were not detected against Vi, which is present in most *S.* Typhi isolates, including the Quailes strain. These results are in agreement with the Maryland challenge studies, which showed considerable increases in flagellin and LPS antibody titers soon after infection (during the incubation

period) but only modest rises in anti-Vi antibody titers (64). Of note, clinical illness and relapse were reported in the Maryland challenge studies to occur at the peak of antibody responses (64). Taken in concert, these results suggest that anti-Vi and other anti-*S.* Typhi-specific antibodies are likely to play a role in protection during natural infection. However, their precise contribution to host defense, either independently or in conjunction with other effector immune responses, remains to be established.

The Maryland CHI studies conducted in the 1950s, 1960s, and 1970s did not address the role of CMI in protection against *S.* Typhi infection, primarily due to the lack of appropriate assays. It is likely, however, that the performance of in depth CMI studies with specimens obtained from subjects participating in the recently re-established Oxford typhoid CHI model using the most advanced current techniques and instrumentation, will greatly advance our understanding of the role of CMI in protection.

TYPHOID AND PARATYPHOID VACCINES: CURRENT STATUS

The first typhoid vaccines consisting of inactivated (heat-killed, phenol-preserved) *S.* Typhi delivered parenterally were developed as far back as 1896 by Pfeiffer and Kolle in Germany and Wright in England (65). At that time, typhoid fever was a much-feared disease. However, following the discovery that antibiotics such as chloramphenicol could successfully treat typhoid fever, the interest in typhoid vaccines waned. A resurgence of interest in typhoid vaccines began in the 1970s, when epidemics of chloramphenicol-resistant typhoid occurred in Mexico and Vietnam (1). Although inactivated whole-cell vaccines are immunogenic and effective, due to excessive reactogenicity, they are no longer manufactured (66–68). Currently, there are two vaccines against *S.* Typhi that are licensed in the USA for use in humans, the purified Vi ("virulence") polysaccharide parenteral vaccine and the oral live-attenuated *S.* Typhi strain Ty21a vaccine. Both vaccines are moderately protective and have been shown to induce herd immunity (69, 70). The Vi polysaccharide vaccine was developed by Robbins and collaborators at NIH as an injectable subunit vaccine and is currently sold by several companies, including Sanofi Pasteur and Glaxo-SmithKline (**Table 1**) (69, 71–75). Although the Vi vaccine confers a moderate level (55–72%) of protection in children over 2 years of age after a single dose, this vaccine does not confer "memory" and there are no robust data to suggest that the efficacy of Vi persists beyond 3 years (66, 67, 69, 76). The Ty21a vaccine, licensed for children older than 6 years, confers a moderate level of long-lived protection (60–80%, 5–7 years) but requires the administration of three to four spaced doses (66, 70, 77). Despite its moderate immunogenicity much of our knowledge regarding immunological responses against *S.* Typhi has been derived from studies of Ty21a immunization (**Table 1**) (52, 66, 67, 78–92). Vaccination of children younger than 2 years old, however, requires a new approach. The Vi-protein-conjugate vaccines appear promising in this regard (14, 93–96). Conjugate Vi vaccines consist of the *S.* Typhi Vi polysaccharide, a T-cell-independent antigen, covalently bound to a carrier protein. Hence, the conjugation process increases the immunogenicity of the vaccine by converting the Vi polysaccharide into a "T-cell-dependent" antigen. Various Vi-conjugate vaccine candidates are in development. For example, Vi *O*-Acetyl Pectin-rEPA conjugate vaccine, a modified conjugate vaccine where Vi is conjugated to non-toxic recombinant *Pseudomonas aeruginosa* exotoxin A (rEPA) has shown an efficacy of ~90% in 2–5-year-old children (94, 96–99). Recently, Bharat Biotech in India has launched the world's first Vi-conjugate vaccine, called Typbar-TCV™, consisting of Vi from *S.* Typhi strain Ty2 conjugated to tetanus toxoid (TT) as a carrier protein, which can be given to infants older than 6 months (100, 101). Other vaccine candidates include Vi-conjugated to CRM_{197} (95) and diphtheria toxoid (102) (**Table 1**). Of note, issues that have been raised and merit consideration regarding the use of Vi and Vi-conjugate vaccines are the emergence of *S.* Typhi Vi antigen-negative strains in multidrug-resistant typhoid fever cases and the possibility that the generalized use of Vi vaccines might lead to increased incidence of enteric fevers caused by Vi-negative strains for which Vi vaccines will be ineffective (103, 104). As a result of these issues, as well as other scientific, logistical, and economic reasons, additional subunit vaccine candidates are being actively developed for the prevention of enteric fevers. These include, among others, conjugates of *S.* Typhi and *S.* Paratyphi A LPS to carrier proteins or *Salmonella* proteins (e.g., flagellin, porins) to extend the generation of immunity to other relevant specific antigens (101).

Because of the above considerations, investigators, including those at the University of Maryland Center for Vaccine Development (CVD), have engineered new attenuated typhoid vaccine strains that aim to be as safe as Ty21a but immunogenic and protective following the ingestion of only a single dose. These vaccine candidates include Ty800 (113), M01ZH09 (114–120), and others based on attenuation of *S.* Typhi by deletions of genes such as those involved in the synthesis of aromatic amino acids (*aroC*, *aroD*) and heat-shock proteins (*htrA*). The latter vaccine candidates, designated CVD 906 (105, 106), CVD 908 (107–109), CVD 908-*htrA* (110), and CVD 909 (112), have been evaluated in volunteers and shown to induce potent CMI both *in vitro* and *ex vivo* (83–85, 105, 107, 110–112, 121–123), as well as humoral responses (105, 108, 110, 112) (see below for details). Except for CVD 906, these strains are derived from the wild-type *S.* Typhi Ty2 strain, the same strain from which the Ty21a vaccine was derived. **Table 1** includes a summary of the characteristics of these typhoid vaccine strains and the documented immune responses elicited in volunteers.

Regarding *S.* Paratyphi vaccines, the first killed whole-cell parenteral typhoid vaccines produced a century ago consisted of a trivalent combination of heat-inactivated and phenol-preserved *S.* Typhi, *S.* Paratyphi A, and *S.* Paratyphi B (TAB vaccine) (67). Although this vaccine was moderately efficacious, its manufacture was discontinued due to high levels of reactogenicity (2). Although several vaccine candidates against enteric fever caused by *S.* Paratyphi A are at various stages of development, including *S.* Paratyphi A O-specific polysaccharide-TT and CRM_{197} conjugates (124–126), no vaccines are currently commercially available.

It is important to note that there has been considerable interest in exploring the use of attenuated *S.* Typhi strains as live-vector vaccines. *S.* Typhi presents multiple advantages as a live-vector, including (a) oral delivery, (b) targeting of M cells overlying gut-associated lymphoid tissue (inductive sites for immune responses), (c) internalization by DC and macrophages, and (d) stimulation of broad immune responses (127). Indeed, multiple clinical

Table 1 | Selected licensed *S.* Typhi vaccines and vaccine candidates.

Type of vaccine	Trade name	Licensed	Manufacturer/ developer	Number of doses	Efficacy (field trials)	Minimum age for administration	Immunogenicity data	Reference
Inactivated whole cell	N/A	Yes	No longer being manufactured	2	~60–80%	N/A	Serum antibodies, lymphocyte proliferation, PBMC migration inhibition	(66–68)
Live attenuated	Ty21a (Vivotif ®)	Yes	Crucell Switzerland Ltd	3–4	~60–80%	≥6 years	Serum antibodies, ASC, ALS, ADCC, opsonophagocytosis, B memory, lymphocyte proliferation, production of multiple cytokines, and chemokines, CTL activity, cross-reactivity with *S.* Paratyphi A & B	(52, 66, 67, 78–92)
	CVD 906	No	CVD–UMB	1	N/A	N/A	Serum antibodies, jejunal IgA, ASC, lymphocyte proliferation, IFN-γ and IL-6 production	(105–107)
	CVD 908	No	CVD–UMB	1	N/A	N/A	IgA ASC, serum IgG, lymphocyte proliferation, IFN-γ and IL-6 production	(107–109)
	CVD 906-htrA	No	CVD–UMB	1	N/A	N/A	Serum antibodies, jejunal IgA, ASC, lymphocyte proliferation	(110)
	CVD 908-htrA	No	CVD–UMB	1	N/A	N/A	Serum antibodies, jejunal IgA, ASC, lymphocyte proliferation, IFN-γ production	(110, 111)
	CVD 909	No	CVD–UMB	1	N/A	N/A	Serum antibodies, ASC, ALS, B memory, opsonophagocytosis, lymphocyte proliferation, cross-reactivity against *S.* Paratyphi A and B	(89, 90, 112)
	Ty800	No	Massachusetts General Hospital	1	N/A	N/A	IgA ASC, serum IgG and IgA	(113)
	M01ZH09	No	Microscience Limited	1	N/A	N/A	Serum antibodies, ASC, ALS, opsonophagocytosis, bactericidal, lymphocyte proliferation, IFN-γ production	(114–118)
	χ3927	No	CVD–UMB	1	N/A	N/A	Serum antibodies, Jejunal sIgA, ASC	(105)
SUBUNIT								
Vi polysaccharide	Typhim Vi®	Yes	Sanofi Pasteur	1	55–72%	≥2 years	Serum antibodies	(66, 67, 72)
	Typherix ®	Yes	GlaxoSmithKline	1	61%	≥2 years	Serum antibodies	(66, 67, 69)
	Typbar®	Yes	Bharat Biotech	1	N/A	≥2 years	Serum antibodies	(73)
	Vax-TyVi®	Yes	Finlay Instituto	1	N/A	≥5 years	Serum antibodies	(74)
	TyViVac	Yes	Dalat Vaccine Company (DAVAC)	1	N/A	≥2 years	Serum antibodies	Product insert
	BioTyph™	Yes	BioMed	1	N/A	≥2 years	Serum antibodies	Product insert

(Continued)

Table 1 | Continued

Type of vaccine	Trade name	Licensed	Manufacturer/ developer	Number of doses	Efficacy (field trials)	Minimum age for administration	Immunogenicity data	Reference
Vi combination	Hepatyrix™ (hepatitis A and Vi)	Yes	GlaxoSmithKline	1	N/A	≥15 years	Serum antibodies	(75)
	ViATIM® (hepatitis A and Vi)	Yes	Sanofi Pasteur	1	N/A	≥16 years	Serum antibodies	(75)
Vi conjugate	Pedatyph™ (Vi-TT)	Yes	BioMed	2	N/A	≥3 months	Serum antibodies	(93)
	Typbar-TCV™ (Vi-TT)	Yes	Bharat Biotech	1	N/A	≥6 months	Serum antibodies	(100, 101)
	Vi-rEPA	No	NIH	2	89%	≥2 years	Serum antibodies	(94, 96, 99)
	Vi-CRM_{197}	No	Novartis Vaccines Institute	1	N/A	18–40 years	Serum antibodies	(95)

N/A, not available; CVD–UMB, Center for Vaccine Development–University of Maryland Baltimore; PBMC, peripheral blood mononuclear cells; ASC, antibody secreting cells; ADCC, antibody dependent cellular cytotoxicity; CTL, cytotoxic T lymphocyte; ALS, antibodies in lymphocyte supernatant; IFN-γ, interferon-γ; (IL6) interleukin-6, NIH, National Institutes of Health; TT, tetanus toxoid; rEPA, non-toxic recombinant Pseudomonas aeruginosa exotoxin A; CRM_{197}, mutant diphtheria toxin carrier protein.

trials have been performed to investigate the immunogenicity of genetically engineered *S.* Typhi expressing foreign antigens (111, 127–134). While these studies have detected only modest immune responses against the foreign antigens, novel engineering strategies hold great potential to enhance the immunogenicity of such vaccines (127). This remains an important avenue of research and improved understanding of immune responses elicited by *S.* Typhi and *S.* Paratyphi A vaccines may facilitate these efforts.

ADAPTIVE RESPONSES TO *S.* Typhi IN VOLUNTEERS IMMUNIZED WITH LICENSED TYPHOID VACCINES AND VACCINE CANDIDATES

As discussed above, immunity to *S.* Typhi is complex involving antibodies and CMI (135–138). Because *S.* Typhi is a facultative intracellular bacterium, we and others have hypothesized that both antibodies and CMI might play complementary roles in protection from infection. While antibodies are likely to play an important role in defense against extracellular bacteria, CMI is expected to be essential in eliminating *S.* Typhi-infected cells. Based on results from studies using specimens from subjects immunized with attenuated typhoid vaccines, we surmise that serum antibodies, SIgA, $CD4^+$, $CD8^+$, and other T-cell subsets (e.g., mucosal associated invariant T-cells, MAIT), as well as the interaction between T, B, and antigen-presenting cells (APC, e.g., macrophages, DC) are all likely to contribute to an effective acquired immune response against typhoid fever (**Figure 1**). However, the relative contribution of each main arm of the effector immune response, i.e., humoral and cellular, and the antigen specificity of the responses remain largely unknown. Below, we will critically address the key humoral and CMI responses, which we believe are essential in generating "protective" immunity against *S.* Typhi infection, as well as discuss current gaps in knowledge, which need to be addressed to enable the identification of immunological correlates of protection in enteric fevers.

HUMORAL RESPONSES

Antibodies

Numerous studies have reported serum antibody production following *S.* Typhi infection and immunization. Antibodies against the O antigen of *S.* Typhi LPS, the Vi antigen, and the H antigen are routinely measured as markers of immunogenicity following *S.* Typhi immunization (67, 110, 112–114, 139, 140). Despite extensive study, the precise role that antibodies play in protection against *S.* Typhi remains unknown. As discussed above, relapses of typhoid fever occur in individuals despite elevated titers of serum anti-*S.* Typhi antibodies (34, 141) and in a recent human challenge with wild-type *S.* Typhi, pre-challenge levels of anti-H and Vi antibodies did not correlate with protection (63). These studies showed that volunteers who were diagnosed with typhoid demonstrated increases in IgG, IgM, and IgA to LPS and H antigens while little change was seen in volunteers who did not succumb to the disease (63). Anti-Vi levels remained unchanged throughout the study (63). Nevertheless, the fact that Vi polysaccharide vaccines can induce protection against typhoid indicates that high-anti-Vi antibodies are protective. In fact, defined levels of serum anti-Vi antibodies (1.4–2.0 μg/ml) have been reported to act as a serological surrogate of protection in Vi-rEPA conjugate vaccine

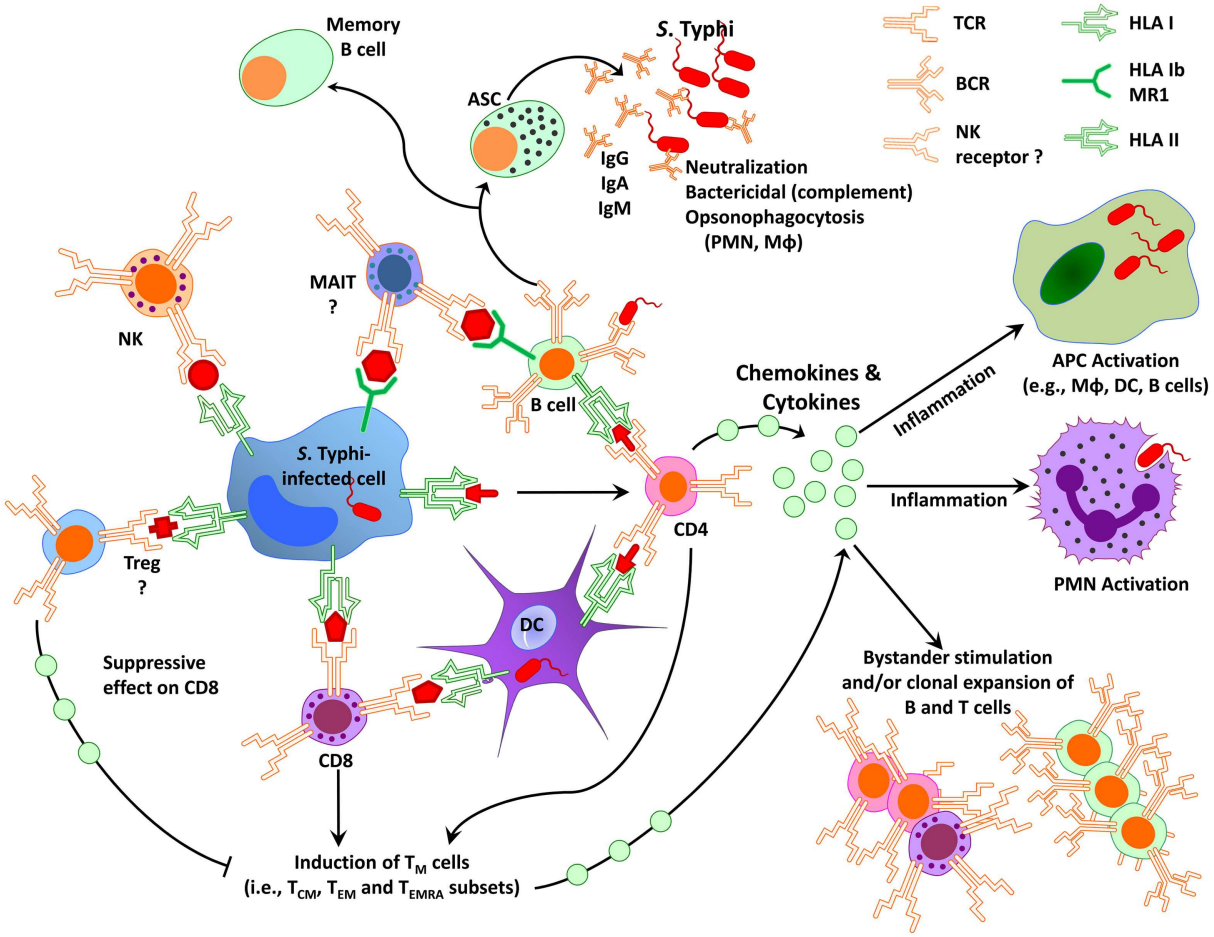

FIGURE 1 | Simplified diagram of immunity to *S.* Typhi in humans. Immunity to *S.* Typhi is extremely complex involving multiple antigen-presenting cells (e.g., macrophages, dendritic cells, B cells) and effector cells (e.g., various effector and regulatory T-cell subsets, B cells, NK, and MAIT cells). APC, antigen-presenting cells; ASC, antibody secreting cells; DC, dendritic cells; CD8, $CD8^+$ T-cells; CD4, $CD4^+$ T-cells; MAIT, mucosal associated invariant T-cells; Mφ, macrophages; NK, natural killer cells; PMN, polymorphonuclear neutrophil; T_M, memory T-cells; T_{CM}, central memory T-cells; T_{EM}, effector memory T-cells; T_{EMRA}, effector memory expressing CD45RA; T_{reg}, regulatory T-cells; HLA, human leukocytes antigen; HLA-I, HLA class I; HLA-II, HLA class II; BCR, B cell receptor; TCR, T-cell receptor; MR1, HLA-I non-classical (b) molecule MR1; Ig, immunoglobulin.

efficacy trials (94). Presumably, anti-Vi antibodies function by counteracting the evasion of innate immune recognition in the intestinal mucosa and obstruction of bacterial-guided neutrophil chemotaxis, which have been proposed as possible mechanisms by which Vi subverts host immune responses (142, 143). Interestingly, the live-attenuated oral vaccine Ty21a, which lacks the Vi antigen, results in similar levels of protection as those of the Vi polysaccharide vaccine (12), indicating that multiple adaptive immunological responses can lead to effective protection (**Table 1**). In field studies of an enteric-coated capsule formulation of Ty21a, seroconversion, as measured by anti-O IgG, correlated with protection (67, 144). However, in these same clinical trials the seroconversion rate of IgG O antibodies did not predict the poor efficacy of other vaccine formulations (67). Seroconversion against *S.* Typhi-O antigen has, nevertheless, been used as a marker of immunogenicity following immunization with single-dose live-attenuated vaccine candidates (110, 112–114, 140). In addition to serum antibodies, *S.* Typhi-specific IgA can be found in saliva, intestinal fluids, and stools following oral immunization with live-attenuated *S.* Typhi or natural infection (26, 78, 145, 146).

Immunoglobulins can be divided into subclasses (e.g., IgA1 and IgA2) based on structural, antigenic, and functional differences (147). The subclasses of IgA are not evenly distributed among bodily fluids with IgA1 dominating in serum and IgA2 found primarily in secretions. In individuals immunized with Ty21a vaccine, *S.* Typhi-specific IgA1 predominated in serum, saliva, and tears, while IgA2 predominated in intestinal lavage fluid (146). IgG can be subclassified into IgG1-4 with different subclasses typically responding to different types of antigen. For example, IgG1 and IgG3 are generally induced by protein antigens, while IgG2 and IgG4 antibodies are associated with polysaccharide antigens (147). Interestingly, however, serum antibodies against *Salmonella* LPS belong primarily to the IgG1, IgA1, and IgA2 subclasses (148). In contrast, as expected, IgG2 anti-Vi was found to be the predominant IgG subclass in a Vi polysaccharide vaccine study

in Nepal (33). Moreover, following a single subcutaneous dose of an *S.* Typhi vaccine candidate containing porins (protein antigen) IgM and both IgG1 and IgG2 seroconversions were detected (136). Unfortunately, no information is available on the avidity of anti-*S.* Typhi antibodies elicited by natural infection or immunization. This is a key measurement of the strength of the attachment of antibodies to their antigen, which is highest after B cells have been adequately primed and is an important measurement of the strength of the anamnestic response. Further understanding of the specific immunoglobulin subclasses and avidity associated with protective responses will be of importance in informing decisions regarding vaccine development.

Despite the large amounts of data regarding production of antibodies against *S.* Typhi, there have been few investigations of the functional properties of these antibodies. Early studies indicated that *S.* Typhi-specific IgA was responsible for antibody dependent cellular cytotoxicity (ADCC) following Ty21a immunization (87). In Nepal, an *S.* Typhi endemic region, bactericidal activity of serum was shown to increase with age; however, no correlation was found between bactericidal titer and anti-Vi titers (33). Recently, we, and others, have reported the induction of functional opsonophagocytic bactericidal *S.* Typhi-specific antibodies that might assist in the elimination of *S.* Typhi (90, 118). These opsonophagocytic antibodies appear to be of the IgG isotype. Further investigation of these functional antibodies may lead to improved measures of immunogenicity and might prove to be more closely associated with protective immunity than antibody measurements by ELISA.

In sum, the sometimes conflicting and fragmentary data regarding the role of antibodies in defense against *S.* Typhi suggest that while they may contribute to an effective response, they are unlikely to represent the dominant mediator of protection in humans following exposure to wild-type organisms.

B cells

Although, studies in knockout mice indicate that B cells play an important role in protection against *S.* Typhimurium (149), the precise role that B cells play in protection against *S.* Typhi in humans remains unknown. Antibody production is clearly a major function of B cells; however, B cells also contribute to immune responses via antigen presentation, cytokine production, and the initiation of T-cell responses. For example, *Salmonella*-specific primary human B cells are able to internalize *S.* Typhimurium via their B cell receptor and stimulate a strong recall response by cytotoxic $CD8^+$ T-cells (150). In fact, following internalization, *Salmonella* survive in the B cell and antigens are loaded onto MHC class I for cross-presentation to $CD8^+$ T-cells (150). These results are supported by our previous observations showing that *S.* Typhi-infected B cells can serve as excellent APC for *S.* Typhi antigens. We reported that Epstein–Barr virus (EBV)-transformed lymphoblastoid B cell lines (B-LCL) are able to effectively stimulate $CD4^+$ cells as well as classical and non-classical $CD8^+$ cells (82–85, 92, 121–123, 151, 152). These findings also re-emphasize the importance of communication among immune cell compartments and the possibility that B cells contribute to host defense from *S.* Typhi infection through mechanisms beyond their primary role in antibody production (20) (**Figure 1**).

Antibody secreting cells. A key aspect of B cells is their ability to undergo cell differentiation and become antibody secreting cells (ASC) (153). In *Salmonella* infection, specific ASC circulate briefly systemically, peaking at ~7–10 days after antigen encounter, before homing to mucosal effector sites (91, 110, 112, 113, 115, 140, 144, 154–157). However, prolonged exposure to antigen results in extended circulation of *S.* Typhi-specific ASC in peripheral blood (158). In fact, patients with prolonged diarrhea have circulating ASC throughout the duration of the pathogen exposure (158). Following mucosal antigen encounter (i.e., oral immunization), *S.* Typhi-specific IgA ASC predominate followed by substantial IgM ASC and low numbers of IgG ASC (79, 158). Of note, the magnitude of ASC response displays considerable inter-individual variation. Three main factors appear to dictate the magnitude of the response: antigen type (live versus killed), number of vaccine doses ingested, and formulation of the vaccine (158). Specifically, immunization with a live oral vaccine resulted in higher magnitude of ASC responses compared to a killed vaccine (159). Ingestion of three doses of vaccine resulted in higher numbers of *S.* Typhi-specific ASC than did two doses and, although there was no further increase in the peak number of ASC following six doses, the response remained higher for a longer duration (158). Additionally, different vaccine formulations (i.e., gelatin capsules, enteric-coated capsules, suspension) showed different magnitudes of response, with the suspension-formulation, resulting in the highest number of *S.* Typhi-specific ASC (79, 158). Notably, the magnitude of the IgA ASC response against the O antigen induced by different formulations and schedules of Ty21a correlated with the efficacy shown in field trials of the same formulations and schedules (79, 144). Other studies showed that the serum antibody increased concomitantly with increasing ASC numbers, and that, when ASC numbers were low, serum antibody responses were undetectable (79). Consequently, it has been proposed that detection of ASC is a more sensitive measurement of immunogenicity than serum antibody titers. The homing patterns of *S.* Typhi-specific ASC have been rigorously studied and are discussed in detail below.

Memory B cells. It is widely accepted that immunological memory is of critical importance for the development of long-lasting protective responses following immunization (160). Memory B cells (B_M) are long-lived antigen primed cells that upon antigenic stimulation during a secondary response undergo rapid terminal differentiation into plasmablasts and plasma cells (161). While there are multiple classification methods to define this heterogeneous population, most B_M are widely accepted to exhibit the phenotype $CD19^+$ $CD27^+$ $IgD^{+/-}$, although a minor B_M subset lacking CD27 expression has also been reported (162). Of note, it has been reported that B_M are able to mature either inside or outside of the germinal centers and that this phenomenon may be T-cell-dependent or independent (161, 163). We have recently made the novel observation that immunization with attenuated *S.* Typhi vaccines elicits $CD19^+$ $CD27^+$ B_M specific for *S.* Typhi antigens (e.g., LPS, flagella, Vi) (89) and described the longevity (up to 1 year), magnitude, and characteristics of these responses (89). Notably, strong B_M responses against both T-cell-dependent (flagella) and T-cell-independent (LPS and Vi) antigens were

identified in volunteers primed with CVD 909 (a Vi expressing live-attenuated *S.* Typhi vaccine candidate). These results suggest that immunization with CVD 909 was capable of mucosally priming the immune system to deliver robust and sustained Vi-specific B_M responses to a subsequent parenteral exposure. LPS-specific B_M responses were also observed in volunteers primed with CVD 909, but these responses were of lower magnitude than those against Vi. Similar to findings for ASC, LPS-specific IgA B_M cells predominated over LPS-specific IgG B_M responses. In the same study, we observed that volunteers immunized with Ty21a also developed IgA B_M responses to LPS, but only a single volunteer developed IgG B_M responses against LPS. Moreover, both CVD 909 and Ty21a were capable of inducing anti-*S.* Typhi flagella IgG and IgA B_M responses. Finally, we observed a strong association between the frequency of antigen-specific B_M cells and antibody levels, supporting an important role of this cell population in the generation of humoral responses. Recent studies have shown that *S.* Typhi porins can induce short- and long-lasting IgG and IgM responses in humans, a response likely to be mediated by B_M (136). Interestingly, studies in mice have also identified IgM B_M, which are likely to secure long-term production of bactericidal IgM antibodies following inoculation with *S.* Typhi porins (164). This study also reported the induction of Type 1 T follicular helper (Tfh) cells that produce IFN-γ, which are thought to support the generation of these B_M (164). However, the relative contribution of the various B_M and Tfh subsets to enduring protection remains to be determined. Further characterization of these responses and cell subsets may help elucidate mechanisms of sustained protection against *S.* Typhi.

B cell phosphorylation. Early signaling events that occur following encounter of B cells with *S.* Typhi and other pathogens are of critical importance in the generation of cellular responses. Recently, we described the phosphorylation patterns associated with *S.* Typhi-specific B cells (165). We reported that exposure of PBMC from healthy volunteers to fluorescently labeled, heat-killed *S.* Typhi resulted in bacterial binding to naïve and unswitched memory (Um) B cells as detected by flow cytometry. Although naïve B cells that interacted with *S.* Typhi were observed, phosphorylation of Stk, Akt, and p38MAPK were not identified in this subset. In contrast, Um B cells showed multi-phosphorylation of all three proteins assayed, as well as cells that phosphorylated only p38MAPK or Akt and p38MAPK. Interestingly, different antigenic structures appeared to induce different patterns of phosphorylation. For example, the phosphorylation patterns induced by *S.* Typhi were dramatically different from the phosphorylation patterns induced by the Gram-positive bacterium *Streptococcus pneumoniae.* These novel studies provide the first glimpse of the activation pathways of *S.* Typhi-specific B cell responses in humans. Further characterization of these mechanisms can provide key information to help advance the generation of novel vaccine strategies.

B cell homing. Although most of our knowledge of immune responses against *Salmonella* in humans is derived from studies using peripheral blood, effector immunity in the local microenvironment of the gut is likely to be of paramount importance in the understanding of protection against *S.* Typhi infection. Mucosal derived circulating IgA ASC detected after administration of live oral typhoid vaccines have been used to estimate the degree of priming of the local intestinal immune system (137). These cells are believed to home to the lamina propria of the intestinal mucosa where they will synthesize and release antibodies (166). Selective homing of cells (including plasmablasts) to the small intestine is believed to be largely driven by the expression of integrin $\alpha_4\beta_7$ and chemokine (C–C motif) receptor (CCR)9 (167), while CCR10 expression appears to be involved in homing to "common" mucosal tissues (168). The primary site of antigen encounter has been shown to affect the expression of homing receptors on ASC (169). Following mucosal antigen delivery by Ty21a administration, robust migration of *S.* Typhi-specific IgM and IgA ASC toward chemokine (C–C motif) ligand (CCL)25 and CCL28, the ligands for CCR9 and CCR10, respectively, were noted (170). In contrast, systemically derived tetanus-specific ASC did not migrate toward either CCL25 or CCL28, supporting the mucosal specificity of these ligands. Previous work has shown that after oral antigen administration, the majority of ASC produce the mucosal Ig-isotype, IgA, and all of them express the gut homing receptor, integrin $\alpha_4\beta_7$, thus, implying mucosal homing of these cells (137, 154, 157, 158). Moreover, when comparing oral Ty21a and parenteral Vi-conjugate vaccines, Ty21a but not Vi immunization recapitulates the homing receptor profile of ASC occurring in natural infection (e.g., integrin $\alpha_4\beta_7$ expression) (155). We have recently shown that sorted IgG and IgA ASC recognizing *S.* Typhi-LPS are predominantly $CD19^+$ $CD27^+$ (a phenotype associated with B_M and plasmablasts) with selective gut homing potential (e.g., integrin $\alpha_4\beta_7{}^+$ $CD62L^-$) (91). Of note, however, both IgG and IgA cells were also observed among integrin $\alpha_4\beta_7{}^+$ $CD62L^+$, suggesting that they have the capacity to home to the gut, as well as peripheral lymph nodes, and perhaps other secondary lymphoid tissues. Further studies of the homing potential of *S.* Typhi-specific B_M and plasmablasts is of critical importance to further our understanding of the mechanisms underlying the induction of antigen-specific cells which have the ability to home to the gut (the initial site of infection), as well as to other lymphoid tissues where *S.* Typhi resides following systemic dissemination.

CELL MEDIATED IMMUNE RESPONSES

As for other intracellular infections, CMI responses against *S.* Typhi infection rely largely on two types of cells: $CD4^+$ and $CD8^+$ T-cells (51, 81, 138, 171). The presence of both $CD4^+$ helper T-cells and classical class Ia and non-classical HLA-E-restricted *S.* Typhi-specific $CD8^+$ T-cells have been observed in individuals with typhoid fever or immunized with Ty21a and other attenuated leading typhoid vaccine candidates, including CVD 908-*htrA* and CVD 909 (51, 82–85, 88, 92, 114, 122, 123, 152, 172). A succinct description of these responses follows.

T-cell responses

We, and others, have reported that *S.* Typhi can stimulate the production of an array of pro-inflammatory cytokines including IFN-γ by specific $CD4^+$ and $CD8^+$ T-cells following immunization (52, 84, 85, 88, 107, 114, 121). For example, IFN-γ production by $CD4^+$ and $CD8^+$ T-cells in response to *S.* Typhi LPS and flagella

antigens has been shown up to 56 days after immunization with attenuated *S.* Typhi vaccines (84, 107, 121). Similarly, in subjects immunized with Ty21a, it has been shown that *S.* Typhi GroEL triggers IFN-γ production by $CD8^+$ cells (85). In addition, *S.* Typhi immunization elicits the generation of cytotoxic $CD8^+$ T-cells (84, 121, 122). Cytotoxic $CD8^+$ T-cells induce apoptosis within minutes of contact with their target by at least two lytic mechanisms (173–175). One, based on granular exocytosis involving perforin and granzymes (176), and another involving a molecule called FAS or APO-1 (177). Using PBMC from individuals immunized with the Ty21a typhoid vaccine (85) and the vaccine candidate strain CVD 909 (123), we have shown that the killing of *S.* Typhi-infected targets by specific $CD8^+$ T-cells is largely through a FAS-independent, granule-dependent pathway. These findings were confirmed using two types of autologous target cells: phytohemagglutinin (PHA)-stimulated PBMC, as well as B-LCL (85, 123). Interestingly, killing of these targets involved antigenic presentation by both classical class Ia and non-classical HLA-E molecules indicating that multiple mechanisms might be involved in killing of *S.* Typhi-infected cells (84, 85, 121).

Cell mediated immunity against *S.* Typhi mediated by $CD4^+$ and $CD8^+$ T-cells appears to depend on the nature of the stimulant. $CD4^+$ cells were more prone to respond to *S.* Typhi soluble antigens while $CD8^+$ cells were more likely to be activated by *S.* Typhi-infected targets (84, 121, 138, 152). These results emphasize the importance of selecting the appropriate type of stimulant when designing experiments aimed at evaluating T-cell responses. Another important issue related to the host's response to *S.* Typhi is the dichotomy between T-cell and humoral responses observed in individual subjects. In the past, our group and others have tried exhaustively, and failed, to observe a correlation on a volunteer by volunteer basis between serum antibody titers to *S.* Typhi LPS and/or *S.* Typhi flagella and CMI in individuals immunized with various attenuated *S.* Typhi vaccine strains (42, 107, 121, 140). These observations support the contention that the development and dominance of humoral and/or CMI responses in individual volunteers is likely multifactorial and influenced by individual host factors (e.g., genetic makeup, gut microbiome composition).

On the basis of the expression of defined surface molecules, T-cells can be simplistically subdivided into two main subsets: naïve and memory T (T_M) cells. Induction of strong and persistent memory T-cell responses is one of the hallmarks of successful vaccination (160, 171). Although T_M can be divided into a multitude of subsets, it is widely accepted that the main T_M subsets are central memory T-cells (T_{CM}), and effector memory T-cells (T_{EM}) (178, 179). T_{CM} express surface molecules for memory (e.g., CD45RO), as well as the chemokine receptor CCR7 and CD62L (L-selectin) molecules, which allow efficient homing to peripheral lymph nodes (178, 179). T_{EM} also express CD45RO, but down-regulate the expression of CCR7 and CD62L, which allows them to circulate and migrate to the spleen and non-lymphoid tissues. In humans, some $CD8^+$ T_{EM} lack the expression of CD45RO and express CD45RA, a molecule present on naïve T-cells. This subset is termed T_{EMRA} or "terminal memory" cells (178, 179). Recently, we provided the first demonstration of the induction and longevity (up to 2 years) of T_{CM}, T_{EM}, and T_{EMRA} multifunctional HLA-E restricted $CD8^+$ T_M cells after Ty21a immunization, suggesting that these cells are important in long-term immunity to *S.* Typhi (82). In these experiments, we showed that following Ty21a vaccination, multiple pro-inflammatory cytokines/chemokines (including IFN-γ) are produced by $CD8^+$ T-cells in response to stimulation with *S.* Typhi-infected targets, and that these responses are multiphasic in nature (82). We also observed a striking correlation among subjects who showed strong $CD8^+$ T_{CM} subsets and produced IL-2 and IFN-γ at early times and the presence of long-term immune responses (82). We speculated that this phenomenon might be due to the fact that IL-2 and/or IFN-γ-secreting $CD8^+$ T_{CM} subsets at early times after vaccination result in the development of a larger pool of long-lived specific $CD8^+$ T_M cell subsets (e.g., $CD8^+$ T_{CM}, T_{EM} and T_{EMRA} subsets), which could lead to improved control against re-infection. Recently, these results were confirmed and extended using multichromatic flow cytometry to measure six cytokines simultaneously (IL-10, IL17A, IL-2, IFN-γ, TNF-α, and MIP-1β) (92). In this work, our group demonstrated, for the first time, the presence of IL-17A-producing $CD8^+$ cells in Ty21a vaccinees (92). These findings are of great significance since consensus is emerging that multifunctional $CD4^+$ and $CD8^+$ T-cells are important in determining the effectiveness of immunity to either vaccination (180) or exposure to intracellular microorganisms in humans, including HIV (181, 182) and *Mycobacterium tuberculosis* (183, 184).

It is important to highlight that the balance between suppressive and pro-inflammatory responses might be of critical importance in the host's ability to mount effective immune responses. For example, experiments in mice have shown that the equilibrium between suppressive T_{reg} and pro-inflammatory T_{eff} responses influence the clearance or persistence of *S.* Typhimurium (185). T_{reg} are characterized by the expression of high levels of the IL-2 receptor (CD25) and transcription factor Forkhead box P3 (FoxP3). Activated T_{reg} may traffic to the sites of specific immune responses and exert their regulatory functions via cell–cell interactions [i.e., cytotoxic T lymphocyte antigen-4 (CTLA-4) competition for co-stimulatory molecules (CD80 and CD86) on APC], consumption of IL-2, and production of anti-inflammatory factors [i.e., IL-10 and transforming growth factor (TGF)-β] (186). Observations in humans, including IL-10 production by PBMC from volunteers immunized with Ty21a and CVD909 in response to *S.* Typhi flagellar antigen (52, 123) and IL-10 detection in the sera of individuals during *S.* Paratyphi A infection (6) indicate a potential role for T_{reg} in establishing a balanced immune response against *S.* Typhi and *S.* Paratyphi infections. Despite these intriguing observations, the role of T_{reg} following *S.* Typhi or *S.* Paratyphi infection or immunization in humans remains unknown.

Background T-cell responses and their possible role in controlling Salmonella infection

A common finding when measuring T-cell immune responses in humans vaccinated against enteric bacteria, such as *S.* Typhi, is the presence of background *S.* Typhi-specific responses among individuals prior to immunization, even in the absence of travel to endemic areas (81, 82, 84, 92, 121, 123, 136, 152). These background responses are characterized by the presence of specific

immune responses against antigens from enteric bacteria in individuals with no history of immunization against, or infection with, the enteric pathogen. Although this background is rather variable, with higher levels observed in individuals in regions with limited sanitation systems (unpublished observations), this phenomenon has been observed in subjects across the World. A prevailing hypothesis is that these background responses are due to the presence of cross-reactive T-cells acquired during previous infections by other enteric pathogens (81, 136, 151) or reacting to the normal gut microbiota (187–190). Although it is difficult to contest these possibilities, it is reasonable to hypothesize that defined subset(s) of T-cells such as innate-like T-cells, including TCRγδ T-cells, NK-T-cells, and MAIT, are responsible, at least in part, for the observed background responses (151). For example, TCRγδ T-cells and NK-T-cells from healthy volunteers with serum antibodies against non-typhoidal *Salmonella* have been reported to produce higher amounts of IFN-γ as compared to conventional $CD4^+$ and $CD8^+$ T-cells in response to stimulation with *Salmonella* antigens (191). It is also known that MAIT cells play an important physiological role in host bacterial defense and may also be involved in inflammatory disorders, particularly at mucosal surfaces (192–194). Previous work has demonstrated that MAIT cells may play a significant role in *M. tuberculosis* and HIV infections in humans. Gold and colleagues have shown in humans that MAIT cells are decreased in the blood of patients with active TB infection. Other reports have shown that the levels of MAIT cells were severely reduced in circulation in patients with HIV-1 infection (195, 196). Their decline was associated with the time of diagnosis (196) and may reflect diverse mechanisms including their accumulation in tissues and activation and functional exhaustion (195, 196). Of note, a recent study from our group has shown that MAIT cells can be activated by B cells infected with various bacteria (commensals and pathogens from the Enterobacteriaceae family, including *S.* Typhi), but not by uninfected cells (151). These responses were restricted by the non-classical MHC-related molecule 1 (MR1) and involved the endocytic pathway. Moreover, the quality of these responses (i.e., cytokine profiles) were dependent on bacterial load but not on the level of expression of MR1 or bacterial antigen on B cell surface (151). Based on these studies, it is reasonable to speculate that baseline responses by functionally active innate-like T-cells (e.g., TCRγδ T, NK-T, MAIT) and/or those elicited early upon microbial stimulation by vaccination or acute infection, might contribute to prevent *S.* Typhi infection. These cell subsets may be responsible for controlling the infection soon after exposure (subclinical infection), and contributing to clear the infection without causing overt disease once the specific adaptive immune responses are fully developed.

Dendritic cell cross-presentation and CD8⁺ T-cells

The mechanism(s) underlying *S.* Typhi regulation of the development of specific T-cell responses in humans remains unclear. Studies in mice have shown that DC can either directly (upon uptake and processing of *Salmonella*) or indirectly (by bystander mechanisms) elicit *Salmonella*-specific $CD8^+$ T-cells (197). DC are APC that have a strategic function in the initiation and modulation of the immune responses (198). In addition to presenting exogenous antigens using the conventional MHC class II activation pathway typically used by $CD4^+$ T-cells, these cells have developed an alternative pathway where exogenous antigens can be presented through an MHC class I activation pathway to $CD8^+$ T-cells (198). This alternative pathway is called the cross-presentation pathway (199). Although multiple APC are able to cross-present antigens, DC are the most efficient *in vivo* (200). Therefore, the successful generation of strong $CD8^+$ T-cell responses to vaccine antigens might be linked to the modulation of the DC cross-presentation.

Our group has provided the first direct demonstration in humans that DC, through suicide cross-presentation, uptake *S.* Typhi-infected human cells and release IFN-γ and IL-12p70, leading to the subsequent presentation of bacterial antigens and triggering the induction of mostly $CD3^+CD8^+CD45RA^-CD62L^-$ T_M cells (201). We observed that upon infection with live *S.* Typhi, human DC produced high levels of the pro-inflammatory cytokines IL-6, IL-8, and TNF-α but low levels of IL-12 p70 and IFN-γ (201). In contrast, DC co-cultured with *S.* Typhi-infected cells produced high levels of IL-12 p70, IFN-γ, and TNF-α (201). These interesting and novel findings are in agreement with previous work showing that IL-12 and IFN-γ are essential for resistance to *Salmonella* infection in mice (21, 202, 203), and that they are likely to also be important in humans (38, 56). Thus, it is reasonable to speculate that cross-presentation of vaccine antigens to $CD8^+$ T-cells might be an important mechanism of antigen presentation leading to the generation of protective immune responses against *S.* Typhi infection.

T-cell homing

Migration or "homing" is a multi-step process where the adhesion of lymphocyte surface homing receptors to their counterparts, addresins, on endothelial cells is the key step (204). As with B cells, the selective homing of effector memory cells to the lamina propria of the small intestine is driven, to a large extent, by the expression of integrin $\alpha_4\beta_7$ and CCR9 (205–209). For example, virtually all T-cells in the small intestine express CCR9 (206). Another molecule implicated in this process is integrin αEβ7 (CD103), which is present in a subset of $CCR9^+$ T-cells (210).

Generation of specific memory $CD4^+$ and $CD8^+$ T-cells with gut homing potential following oral typhoid immunization has been well described (81, 83, 152). Previous work has shown that sorted integrin β7-expressing memory T-cells ($CD45RA^-$ $\beta7^{high}$ cells) from volunteers immunized with *S.* Typhi vaccine strain Ty21a when stimulated *in vitro* produced around 10-fold more IFN-γ than the remaining populations ($CD45RA^-$ $\beta7^-$ or $CD45RA^-$ $\beta7^{intermediate}$) (81). Also, using cells from volunteers immunized with the candidate *S.* Typhi vaccine strain CVD 909, our group further characterized the gut homing potential and induction of IFN-γ production in the central (T_{CM}, $CD45RO^+$ $CD62L^+$) and effector (T_{EM}, $CD45RO^+$ $CD62L^-$) memory T populations (152). Interestingly, we observed that the homing potential of $CD4^+$ and $CD8^+$ T_M subsets were distinct. Although both $CD4^+$ T_{EM} and T_{CM} populations produced IFN-γ, $CD4^+$ T_{CM} cells were predominantly integrin $\alpha_4\beta_7^+$ while $CD4^+$ T_{EM} were found to include both integrin $\alpha4\beta7^+$ and integrin $\alpha4\beta7^-$ cells. In contrast, IFN-γ-producing $CD8^+$ cells were predominantly classical T_{EM} and $CD45RA^+$ T_{EM} (T_{EMRA}; $CD45RO^-$

CD62L$^-$) subsets. Interestingly, while CD8$^+$ T_{EM} included both integrin α4β7$^+$ and integrin α4β7$^-$ cells, CD8$^+$ T_{EMRA} were predominantly integrin $\alpha_4\beta_7^+$ (152). By using PBMC from healthy adults immunized with the Ty21a vaccine, we have also reported that *S.* Typhi-specific CD8$^+$ T-cells are able to co-express high levels of integrin $\alpha_4\beta_7$, intermediate levels of CCR9 and low levels of CD103 (83). Furthermore, we showed that these specific memory CD8$^+$ T-cells with gut homing potential bear multiple TCR Vβ specificities (e.g., Vβ2, 3, 8, 14, and 17) (83). Of note, cells used in this study were collected 5–40 months after oral immunization. Thus, *S.* Typhi-specific CD8$^+$ T_{EM} cells with gut homing potential might persist in circulation over long periods of time. However, because the study used cells isolated exclusively from peripheral blood, we have to consider the possibility that these observations might not reflect the full spectrum of TCR Vβ usage by *S.* Typhi-specific CD8$^+$ T-cells in the gut microenvironment *in vivo*. Based on these findings regarding the homing potential of *S.* Typhi-specific cells, it is reasonable to speculate that the observed multiphasic kinetics of the T-cell responses described above might represent decreases in circulating *S.* Typhi-specific T-cells as they home to the gut and other lymphoid tissues, as well as increases due to the release into the circulation of new waves of specific cells generated in lymphoid organs.

MICROBIOTA, CO-INFECTIONS, AND THE HOST IMMUNE RESPONSE FOLLOWING IMMUNIZATION WITH ORAL ATTENUATED TYPHOID AND OTHER ENTERIC VACCINES

There is growing evidence from clinical studies indicating that the gut microbiota has a profound impact in modulating human immune responses in health and disease, including a significant role in influencing vaccine efficacy (190, 211–213). For example, in a study evaluating the oral attenuated *V. cholerae* O1 vaccine CVD 103-HgR, Lagos and colleagues demonstrated that excessive bacterial growth ("tropical enteropathy") in the small intestine of children in less developed countries might contribute to the low-antibody response to the vaccine (214). In this study, an inverse association was found between bacterial over growth and seroconversion as determined by vibriocidal titers. Reduced vaccine efficacy and immunogenicity in developing countries when compared with North Americans also has been reported with other vaccines, including oral polio and rotavirus (137, 213, 215). Helminth infections have also been demonstrated to impact vaccine immunogenicity and, for example, anti-helminthic therapy prior to immunization was shown to improve the immune response to the CVD 103-HgR cholera vaccine (216). Regarding *S.* Typhi, recent evidence showed that the induction of *S.* Typhi-specific IgG LPS antibodies following immunization was significantly higher among CVD 908-*htrA* vaccines infected with *Helicobacter pylori* than in uninfected subjects. These results are likely the consequence of gastric acid hyposecretion due to *H. pylori* infection which facilitated the passage of CVD 908-*htrA* through the stomach (217). These observations are supported by reports indicating that the risk of developing typhoid fever is higher in *H. pylori*-infected individuals in underdeveloped countries (218), suggesting that the success of the Ty21a typhoid vaccine in endemic regions might be the result, at least in part, of the high prevalence of *H. pylori* infection accompanied by hypochlorhydria (217, 219). Additionally, evidence in animal models suggests that modulation of the gut microbiota (e.g., with antibiotics, prebiotics, and probiotics) can enhance vaccine efficacy (220, 221).

We recently initiated studies to directly investigate the interactions between the microbiome and vaccination with attenuated oral vaccines. We observed that, although Ty21a is a live-attenuated *S.* Typhi vaccine delivered via the oral route, there was no disruption in the composition, diversity, or stability of the fecal microbiota in healthy adult volunteers who received this vaccine (172). However, categorical analysis based on multiphasic CMI responses versus late CMI responses identified a subset of bacterial operational taxonomic units (OTUs) differentiating individuals capable of mounting distinct immunological responses. Generally, individuals who exhibited a multiphasic CMI response to vaccination harbored greater community richness and diversity compared to individuals with only a late CMI response to Ty21a. No differences were identified in community richness or diversity among volunteers characterized as responders or non-responders based on seroconversion (*S.* Typhi LPS). Although the number of volunteers analyzed was small, this study provides additional information supporting the potential influence of the gut microbiota on the immune response elicited by oral immunization, and perhaps, in protection. Additional studies involving larger numbers of volunteers and a multiplicity of vaccines administered via the oral route are necessary to extend our understanding of the complex role of the gut microbiota in modulating host immunity and vaccination in humans, and its possible role in vaccine efficacy.

CROSS-REACTIVE IMMUNE RESPONSES AMONG *S.* Typhi, *S.* Paratyphi A, AND *S.* Paratyphi B

As discussed above, limited information is available regarding host immune responses to *S.* Paratyphi A and *S.* Paratyphi B in humans. In fact, most of the immune responses believed to be elicited by *S.* Paratyphi A have been inferred from *S.* Typhi studies. Interestingly, field trials of Ty21a have shown modest cross-protection against *S.* Paratyphi B (3), suggesting that cross-reactive immune responses might be responsible. The presence of cross-reactive responses were first reported in the 1980s by Tagliabue et al. who reported the induction of IgA antibodies following oral immunization with Ty21a, which mediate T-cell-dependent ADCC against *S.* Typhi, *S.* Paratyphi A, and *S.* Paratyphi B, but not against *S.* Paratyphi C (87). We have recently identified cross-reactive immunological responses against *S.* Paratyphi A and *S.* Paratyphi B in subjects orally immunized with Ty21a (91). IgA ASC that recognized LPS from *S.* Paratyphi A and *S.* Paratyphi B were observed, but at a lower magnitude than responses against *S.* Typhi LPS (91). These cross-reactive anti-LPS CD19$^+$ CD27$^+$ IgG and IgA ASC displayed the same homing pattern (i.e., a dominant integrin $\alpha_4\beta_7^+$ CD62L$^-$ subset and a significant proportion of integrin $\alpha_4\beta_7^+$ CD62L$^+$ cells) as *S.* Typhi-specific ASC. We also reported the induction of antibodies and B_M to *S.* Typhi LPS and OMP antigens, which cross-react with *S.* Paratyphi A and *S.* Paratyphi B. However, IgA B_M reactive to *S.* Typhi was of higher magnitude than those against *S.* Paratyphi A and *S.* Paratyphi B. In contrast, B_M to outer membrane proteins (OMP) from *S.* Paratyphi B were similar to those observed for *S.* Typhi-OMP, but higher than those for *S.* Paratyphi A OMP. In

a subsequent study, we reported in Ty21a and CVD 909 vaccines the presence of cross-reactive serum antibodies able to mediate opsonophagocytosis of *S.* Paratyphi A and *S.* Paratyphi B, albeit at lower levels than those against *S.* Typhi (90, 91). Similar observations regarding cross-reactive ASC responses among *S.* Typhi and *S.* Paratyphi serovars A, B, and C were recently reported in Ty21a vaccinees and patients with enteric fevers (222). These cross-reactive responses are likely the result of the immunity elicited by O:12, the trisaccharide (mannose–rhamnose–galactose) repeating unit that comprises the LPS backbone, which is common to *S.* Typhi, *S.* Paratyphi A, and *S.* Paratyphi B. Of note, a recent study showed that, although *S.* Paratyphi A and *S.* Paratyphi B do not possess the Vi antigen, cross-reactive ASC were identified in recipients of the Vi polysaccharide vaccine (223). The authors concluded that this low level of cross-reactivity is likely attributable to *S.* Typhi-LPS contamination of the Vi polysaccharide vaccine. Similar observations were reported by others (89, 139). Of note, although to our knowledge there are no reports documenting cross-protection against non-typhoidal *Salmonella* in Ty21a or Vi vaccinees, these typhoid immunizations elicit cross-reactive ASC against non-typhoidal *Salmonella*, including *S.* Typhimurium and Enteriditis that share either O:9, O:12, or both antigens with *S.* Typhi (224, 225). In spite of these studies, the precise immune mechanism(s) of the cross-protection observed against *S.* Paratyphi B in Ty21a vaccinees in field trials remains unclear. However, it is tempting to speculate that CMI responses might play a key role in cross-protection. Further studies assessing the basis for these cross-reactive responses, as well as whether immunization with novel attenuated *S.* Paratyphi A vaccines, or wild-type *S.* Paratyphi A infection, results in cross-reactive humoral and CMI responses with *S.* Typhi and *S.* Paratyphi B will provide critical information to advance the development of broad-spectrum vaccines to protect against enteric fevers.

"OMICS" STUDIES

Recent advances in microarray and proteomics technologies have allowed for detection of immunogenic *S.* Typhi antigens (226, 227). Both immunoaffinity proteomics-based technology and protein microarrays have been utilized to identify key antigens that may be suitable for vaccine development and diagnostics (226, 227). Furthermore, transcriptional profiling in peripheral blood of patients infected with *S.* Typhi identified a distinct and reproducible signature that changed during treatment and convalescence (228). Additionally, studies performed in mice and humans have also identified immune signatures common to murine and human systemic salmonellosis (229). Although very few manuscripts have reported the use of these state-of-the-art approaches, these comprehensive analyses of the transcriptional and proteomic profiles provide a foundation for more directed analyses that may have a direct impact on the development of novel vaccines and diagnostics in coming years.

CONCLUDING REMARKS

Despite decades of effort, the mechanisms of protective immunity in natural infection and vaccination remain largely undefined and many questions remain (**Box 1**). The vast majority of the information currently available using modern immunological techniques has been obtained using specimens from subjects immunized with attenuated typhoid vaccines. Old challenge studies lacked the appropriate tools to monitor immune cells (e.g., B- and T-cells) and in general, have been limited to measurements of serum antibody titers and, in some cases, the use of inadequate CMI methodology available at that time. The "Renaissance" of challenge studies with wild-type *S.* Typhi, such as those being performed in Oxford, is at hand and novel technologies to analyze in unprecedented depth the host immune responses have recently become available. One of these technologies is mass cytometry, also known as Cytometry by Time Of Flight (CyTOF), capable of resolving more than 35 measurements per cells using rare metal-conjugated monoclonal antibodies with minimal signal overlap (230–232); a problem that severely limits the number of parameters, which can be evaluated by conventional flow cytometry. This novel technology will enable the simultaneous measurement of the phenotype and function of multiple immune cell types by simultaneously monitoring the cross-talk between traditional players (e.g., B- and T-cells), and new potential players (e.g., innate-like T-cells, including TCRγδ T-cells, NK-T-cells, and MAIT cells, as well as T_{reg} cells) and the possible mechanisms leading to protection against infection. In fact, it is likely that it is the balance (i.e., homeostasis) between effector and regulatory responses that holds the key to understanding protective immunity. Mass cytometry, in conjunction with traditional immunological assays and state-of-the-art genomics, transcriptomics, proteomics, and metabolomics approaches and the availability of human challenge models provide, for the first time, the necessary tools to uncover the mechanisms underlying protective immunity, both systemically and

Box 1 | Key remaining questions.

- What are the relative contributions of humoral and cellular responses to protection?
- What are the precise roles of effector and memory B and T-cells, as well as innate immune cells in protection?
- How can an appropriate balance between pro-inflammatory and regulatory responses be achieved, resulting in protection without causing excessive inflammation?
- What are the mechanisms of enduring protection against *S.* Typhi, *S.* Paratyphi A, and *S.* Paratyphi B and how can long-lasting responses be preferentially induced?
- What are the characteristics of protective local gut immune responses?
- What are the differences and similarities between local and systemic immune responses?
- What is the role of the gut microbiota in modulating immune responses against enteric fevers?
- Can cross-reactive immune responses between *S.* Typhi, *S.* Paratyphi A, and *S.* Paratyphi B be exploited to develop broad-spectrum vaccines against enteric fevers?

in the gut microenvironment. This information will be invaluable in accelerating the development of novel vaccine strategies to prevent enteric fevers. In addition, the expected explosion of knowledge regarding the gut microbiome and its role in modulating immunity to oral vaccines is also likely to provide significant insights in coming years in understanding the observed differences in immunogenicity between vaccine responses in developed and developing countries.

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Identification of protective antigens for vaccination against systemic salmonellosis

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There is an urgent medical need for improved vaccines with broad serovar coverage and high efficacy against systemic salmonellosis. Subunit vaccines offer excellent safety profiles but require identification of protective antigens, which remains a challenging task. Here, I review crucial properties of *Salmonella* antigens that might help to narrow down the number of potential candidates from more than 4000 proteins encoded in *Salmonella* genomes, to a more manageable number of 50–200 most promising antigens. I also discuss complementary approaches for antigen identification and potential limitations of current pre-clinical vaccine testing.

Keywords: *Salmonella enterica*, protective immunity, mouse model, human clinical trials, antigen expression, immunodominance, typhoid vaccines

INTRODUCTION

Salmonella enterica serovars Typhi and Paratyphi A, B, and C cause human enteric fever with an estimated annual number deaths of 190,000 (1). Enteric fever disease burden is probably underestimated because of difficult and insensitive diagnosis methods (2). In addition to these serovars, specific strains of serovar Typhimurium, which usually causes self-limiting gastroenteritis, can also cause systemic disease, particularly in young HIV-infected children in sub-Saharan Africa (invasive non-typhoidal salmonellosis, iNTS) (3).

Enteric fever and iNTS become increasingly difficult to treat with antibiotics because of rising resistance to fluoroquinolones and cephalosporins, and new drug candidates for these and other Gram-negative pathogens are scarce suggesting a risk of an increasing number of untreatable cases (2, 4).

THE NEED FOR NOVEL SUBUNIT VACCINES

Enteric fever can be prevented with a variety of vaccines (5). Killed whole-cell preparations of serovars Typhi and Paratyphi were successfully used to diminish incidence in endemic areas, but their use was discontinued because of frequent adverse reactions (6). A live attenuated *S.* Typhi strain Ty21a that was generated by chemical mutagenesis confers a moderate level of protection for up to three years against serovar Typhi, but not other relevant serovars (6). Additional genetically modified *Salmonella* strains have been tested in clinical trials with some success, but none of them has yet reached approval. Finally, the purified capsular carbohydrate Vi of serovar Typhi induces protective immunity over several years against serovars Typhi (6) and possibly Paratyphi C, but not Paratyphi A and B or Typhimurium that all lack such a capsule. Conjugation of Vi with an unrelated protein antigen improves immune response in small infants, a major target population for enteric fever (6). To cover the important serovar Paratyphi A, current efforts focus on linking the O antigen (carbohydrate part of lipopolysaccharide) with a protein antigen (7).

In conclusion, treatment of systemic salmonellosis becomes increasingly difficult, and prevention with currently available vaccines is hampered by only moderate levels and limited duration of protection, and incomplete coverage of clinically relevant serovars. This situation generates an urgent medical need for improved *Salmonella* vaccines.

Live attenuated *Salmonella* strains offer important advantages such as low production costs and oral administration, but pose a risk of causing disease especially in immunocompromised patients that might be inadvertently exposed, e.g., household contacts of vaccines that shed live *Salmonella*. Whole-cell killed vaccines are effective but contain pyogenic components that cause unacceptable inflammatory responses. As a consequence, development focuses on subunit vaccines that contain one or several key antigens inducing protective immune responses.

The key challenge of developing such a vaccine is identification of suitable antigens. Unfortunately, among thousands of potential *Salmonella* antigen candidates, probably only very few have the necessary properties. Efficient strategies to identify protective antigens among large number of candidates have been developed and applied for vaccines that protect against extracellular pathogens using inhibitory/bactericidal antibodies (reverse vaccinology) (8). For these pathogens, suitable antigens need to be surface exposed to enable antibody binding, which substantially narrows down the number of potential candidates. Furthermore, immunization trials can be scored for inhibitory/bactericidal antibodies using rather simple assays amenable for high-throughput.

In contrast, similar strategies have not yet been developed for intracellular pathogens like *Salmonella* (which reside mostly in host macrophages during systemic disease), since criteria for preselecting promising antigens are unclear for most such pathogens, and immune correlates of protection remain poorly characterized. Antibodies (or just B cells) often contribute to protection but T cell responses are usually also required. The crucial αβ T cells recognize peptide epitopes and this led to a focus on protein antigens. Most intracellular pathogen genomes encode thousands of proteins, and identification of the few protective antigens among these numerous candidates remains challenging.

However, extensive recent work on *Salmonella* has uncovered some information that might be useful as a rational basis for future vaccine development against this and possibly other intracellular pathogens. In particular, coverage of relevant *Salmonella* strains, antigen expression in infected host tissues, and antigen compartmentalization within the *Salmonella* cell may substantially narrow down the number of promising antigen candidates.

ANTIGENS ENABLING BROAD SEROVAR COVERAGE

To achieve protective immunity against all relevant *Salmonella* strains, conserved antigens must be used. Hundreds of genes are missing or dysfunctional due to frameshift mutations or premature stop codons in certain relevant strains (9), but the rapidly increasing collection of genome sequences facilitates identification of suitable broadly conserved antigens. Orthologs usually share extensive sequence identity, but rare non-synonymous point mutations might still affect potentially crucial immunity determinants such as surface-exposed loops of outer membrane proteins (10). The 3D structures of many *Salmonella* proteins have been determined, and additional structures can be modeled based on homologs. However, it remains challenging to estimate which amino acid differences might impair cross-protective immune responses. As a consequence, antigens with highly conserved sequence among relevant serovars might be prioritized. On the other hand, antigens that play a potentially crucial role in pathogenesis of only a subset of serovars such as typhoid toxin (11) could still be an important contributor to vaccine combinations containing multiple antigens.

ANTIGEN EXPRESSION IN HOST TISSUES

To detect and kill *Salmonella*, the immune system must recognize antigens that *Salmonella* expresses in infected host tissues. For animal infection models, purification of genetically engineered fluorescent *Salmonella* cells from infected tissue homogenates using flow cytometry yields sufficient material for large-scale proteome analysis (12, 13). The results reveal expression of more than 1800 *Salmonella* antigens in mouse spleen. As a caveat, this analysis misses most secreted *Salmonella* proteins that are lost during purification. This is important, since at least one secreted protein can confer moderate protection (14). While escaping proteomics of purified *Salmonella*, highly expressed secreted proteins can be identified based on transcriptional *in vivo* data (15, 16).

Recent advances in proteomics enable even absolute quantification of copy numbers per *Salmonella* cell for most detected antigens (10). High expression levels might facilitate immune recognition (10, 15), but our systematic analysis did not support that protective antigens are generally highly expressed (10). This could reflect extensive host–pathogen coevolution modulating expression levels and immunogenicity of antigens. However, despite the poor predictive power of quantitative expression levels, antigen expression itself remains a crucial precondition for protective immune responses.

Salmonella proteomes in human tissues have not yet been investigated. However, experimental infections of human volunteers have been done in the past (17, 18), and a well-controlled protocol has recently been established (19). Purification by flow cytometry similar to the mouse studies would require infection with a genetically modified *Salmonella* strain, and type and required quantities of biopsy material would need to be determined.

Salmonella virulence has been extensively characterized in the mouse typhoid fever model. These studies have identified more than 270 *Salmonella* genes that contribute to pathogenesis. In almost all cases, this evidence indicates expression of the respective antigens at least at some stage of the infection. Virulence phenotypes in human beings are also available in a few cases from vaccine trials with live attenuated *Salmonella* strains (20–22). These scarce human data are largely consistent with observations for the corresponding *Salmonella* mutants in the mouse model, but systematic comparisons are currently impossible due to the lack of human data for most potential virulence factors.

Using another indirect approach, large-scale studies have identified antibodies that specifically recognize dozens of *Salmonella* antigens in sera of acutely infected and convalescent patients or experimentally infected mice, but not uninfected controls (23, 24). The presence of such antibodies is a clear indication that the respective *Salmonella* antigens are expressed at least at some stages of infection. Interestingly, there is a considerable overlap in immune signature of murine and human salmonellosis. On the other hand, comparison with direct *ex vivo* proteome analysis of *Salmonella* purified from infected mouse spleen reveals that serum antibodies recognize only a small minority of the more than 1800 *in vivo* expressed *Salmonella* antigens. It is possible, that *Salmonella* antigens that induce specific antibodies are particularly accessible for the host immune system, and thus represent most promising vaccine antigen candidates. However, direct comparison of antibody titers in convalescent mice with antigen protectivity in immunization/challenge studies shows that serum antibody levels have poor predictive power for identifying suitable vaccine antigens (10, 23). In fact, several of the most protective vaccine antigens failed to elicit detectable antibody responses in both mice and human beings, while immunodominant antigens mostly fail to protect.

Similar to antibody response in convalescent individuals, T cell responses to specific *Salmonella* antigens provide information about antigen expression during infection. CD4 T cell epitopes have been comprehensively predicted based on peptide properties that facilitate binding to antigen-presenting major histocompatibility complex (MHC) II molecules and T cell receptors (25). Some antigens were experimentally confirmed to be recognized by T cells from infected human beings (26, 27) and mice (14, 25, 28, 29), but not uninfected individuals. These results confirmed expression of corresponding *Salmonella* antigens (including the promising antigen SseB) at least during some stages of infection. Again, these

identified antigens are only a small subset of all expressed antigens, and T cell responses during infection have poor predictive power for protective antigens (10). This might reflect expression at infection stages (29) or in distinct tissue microenvironments (30) that have limited relevance for protective immunity.

In conclusion, proteomics and virulence phenotypes provide large-scale information on *Salmonella* antigen expression in infected mice. Together, some 2000 different *Salmonella* antigens are expressed during infection in the mouse typhoid fever model, and might thus represent potential vaccine antigens. Evidence for human infections is much more fragmentary and largely restricted to serum antibody and T cell responses.

ANTIGEN COMPARTIMENTALIZATION

The localization of an antigen within the *Salmonella* cell may have a major impact on its protectivity. In particular, live intact *Salmonella* can only be detected by the host immune system through recognition of surface-exposed/released antigens, since internal *Salmonella* antigens are shielded by the cell envelope. On the other hand, dead *Salmonella* might release antigens regardless of their initial localization. In many infection foci, live and dead *Salmonella* reside in close proximity (30), and recognition of dead *Salmonella* alone might be sufficient for activation of bystander cells containing live *Salmonella*, resulting in effective clearance of both live and dead *Salmonella*. However, a subset of live *Salmonella* resides in tissue regions without any dead *Salmonella* (10, 30), and these would escape detection/clearance by immune responses directed exclusively against internal *Salmonella* antigens. This working model is supported by previously identified protective antigens (15, 31–33) and our systematic comparison of *Salmonella* antigens from different compartments (10): all identified protective antigens are surface exposed. A recent study extended this finding to a secreted virulence effector protein (14), supporting the hypothesis that antigens must be accessible on live *Salmonella* to confer protective immunity.

Surface exposure/secretion might represent a powerful criterion to narrow down the number of potentially promising *Salmonella* vaccine antigens. Surface-exposed outer membrane proteins can be identified based on primary sequence properties and have been tabulated in databases (34, 35). Interestingly, outer membrane-associated lipoproteins can also confer protective immunity, even when they likely localize to the shielded periplasmic side of the outer membrane (10). Possibly, such lipoproteins are released in outer membrane vesicles that are degraded in host cell lysosomes thus exposing lipoproteins to the antigen-presentation platforms. Outer membrane-associated lipoproteins can again be identified based on primary sequences (36). Experimental analysis of outer membrane preparations (37, 38) and/or biotinylated surface-exposed proteins (39) can be used to confirm theoretical predictions, and to identify additional exposed antigens that might be secreted through unconventional mechanisms.

In addition to surface-associated proteins, *Salmonella* translocates various proteins directly to the infected host cell cytosol, predominantly using the SPI-2 associated type III secretion system. SPI-2 effector proteins are intensively studied and the currently identified list of 32 proteins (40) might approximate completion. The SPI-2 translocon subunit SseB itself is one of the most promising vaccine antigens (15, 23, 27). During initial phases of infection, *Salmonella* secretes proteins also through the SPI-1 associated type III secretion system and through the flagellar apparatus (in particular, flagellin, a moderately protective antigen) (29, 31).

Together, surface-exposed and secreted *Salmonella* antigens comprise some 200 different antigens, and at least around 50 of them are expressed during infection in the mouse typhoid model based on transcriptional data, proteomics, virulence phenotypes, and/or immunization data. Twenty-six such antigens have already been tested and nine appear to confer some degree of protective immunity in mouse typhoid fever immunization/challenge studies (FliC, SseB, OmpD, CirA, IroN, T0937, SlyB, PagN, and SseI; in some cases group sizes were too small to obtain definitive proof) (10, 14, 15, 29, 31–33).

FUTURE PERSPECTIVES

Immunization/challenge experiments in the mouse typhoid fever model have shown that live attenuated *Salmonella* strains can provide full long-term protection against otherwise lethal challenge infections (41). Compared to this benchmark, progress with subunit vaccines in the same model has been somewhat disappointing. Despite large-scale experimental and computational screening campaigns in several different laboratories, few *Salmonella* antigens with at most moderate protectivity in the mouse typhoid fever model have been identified. None of these antigens confer full protection for more than some 30 days after challenge infection. This could reflect immune evasion of the challenge *Salmonella* strain by mutation of crucial epitopes within the respective antigens. During such a long infection time, other adaptive immune responses might be expected, but these responses are obviously insufficient for protective immunity.

It is possible that the best protective antigens have not yet been identified, or that multiple antigens need to be combined for full protection and prevention of immune evasion. It is also possible that antigens other than proteins, such as lipids, carbohydrates, or even small molecules (42), are necessary for high levels of protection. One approach to test this hypothesis could use progressive depletion of specific antigens from protective live attenuated *Salmonella* strains, by deleting respective biosynthesis genes. However, this approach is limited to non-essential genes and is thus non-informative for antigens such as riboflavin intermediates (12, 42). Alternatively, killed whole-cell vaccines might be fractionated and tested for protection. Unfortunately, killed whole-cell vaccine formulations with high protective efficacy in the mouse typhoid fever model have not yet been described. Future studies might revisit this issue, especially since killed whole-cell vaccines confer substantial protective immunity against invasive salmonellosis in human beings (although they are no longer used because of severe adverse reactions) (1).

Finally, it is important to consider what level of protection is actually needed in pre-clinical mouse models before proceeding to human clinical vaccine trials. In the typhoid fever model, genetically high-susceptible mouse strains defective for the divalent cation transporter *Slc11a1* (*NRAMP1*) (43), are infected with doses of *S. enterica* serovar Typhimurium that result in an attack rate of 100%. This combination reproduces some important aspects of human disease including *Salmonella* dissemination from intestinal

sites, histopathology in spleen and liver (splenomegaly, formation of structured inflammatory lesions), relevance of various *Salmonella* virulence factors and host cytokines, and protective immunity against reinfection in convalescent individuals, or individuals vaccinated with live attenuated *Salmonella* strains (41). On the other hand, disease progression in mice is more rapid and always lethal when using wild-type *Salmonella* strains, in contrast to human enteric fever. Importantly, protective immunity against challenge infections in the mouse model requires both B cell and CD4 T cell responses (28), but neither antibodies (44) nor MHC I-restricted CD8 T cells (45). In contrast, vaccination-induced antibodies alone seem to confer already a substantial level of protection in human beings (46), at least in endemic areas where pre-existing immune responses to *Salmonella* are highly prevalent (24). It is thus possible that full protection against virulent wild-type *Salmonella* strains in genetically susceptible mice might be too stringent a criterion to judge vaccine efficacy.

Instead, it might be worth considering genetically resistant mice (47), in which antibodies seem to suffice for protective immunity (48), and heat-killed *Salmonella* mediate substantial immunity (49) similar to the situation in human beings (6). Interestingly, flagellin is also highly protective in resistant mice (50), in contrast to only moderate protectivity in susceptible mice.

In addition to the mouse strain, the challenge infection dose should be re-considered. Controlled human trials have shown that vaccine-induced immunity can be easily overwhelmed by even moderate challenge doses (17). Vaccines that have well-documented efficacy in field-trials, completely fail to protect against *S.* Typhi when given at doses in the range of 10^6–10^7 CFU. Vaccine efficacy is only seen at a much lower dose of 10^5 CFU that caused disease in only 40% of unvaccinated control volunteers [a recent study showed higher attack rates at such doses (19)]. Based on these human data, commonly used mouse challenge infections that result in 100% attack rates might be too stringent for revealing a moderate level of protective immunity that could still be sufficient for preventing even a large proportion of human disease under relevant field conditions.

Finally, a better understanding of human immune responses that are relevant for protective immunity could help to replace the crude readout parameter "survival after challenge infection" with more informative quantitative immune parameters. Ongoing studies in an experimental human infection and vaccination model (19) will likely provide such crucial information in the near future.

CONCLUSION

Several *Salmonella* antigens that can mediate at least partial protective immunity against lethal challenge infections in mice have recently been identified. Analysis of their properties suggests that efforts to identify further suitable antigens might focus on a limited number of promising surface-associated/secreted candidates that are expressed in infected host tissues. However, none of the known individual antigens mediates solid strong protection, comparable to what can be achieved with attenuated live *Salmonella* strains. Future studies could explore antigen combinations and possibly antigens other than proteins. Moreover, a better understanding of qualitative and quantitative immune parameters that are required to protect human beings is needed to guide pre-clinical models for further vaccine optimization and to determine what levels of protection are needed.

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Why the development of effective typhoid control measures requires the use of human challenge studies

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INTRODUCTION

Salmonella Typhi (*S.* Typhi) has affected mankind for the last 50,000 years (Kidgell et al., 2002; Roumagnac et al., 2006), however the precise pathogenesis in humans has largely remained a mystery (Crump and Mintz, 2010). Typhoid fever, the systemic disease caused by *S.* Typhi infection, is responsible for an estimated 21 million new infections annually resulting in approximately 200,000–600,000 deaths world-wide (Crump et al., 2004; Buckle et al., 2012). If untreated, typhoid fever may result in severe illness including the complications of gastrointestinal bleeding, bowel perforation, and sometimes death. Transmission of *S.* Typhi occurs via ingestion of faecally-contaminated food or water (De Jong et al., 2012). Infection risk has been associated with household factors including contact with a recently infected relative, poor sanitation and hygiene infrastructure, which include spatial associations with contaminated public water sources (Vollaard et al., 2004; Sur et al., 2009; Baker et al., 2011).

Much of what we know about human *Salmonella* infection has been determined from historical human vaccine and, subsequently, challenge studies dating back to 1896 (Waddington et al., 2014a). Between 1952 and 1974, human challenge studies performed at the University of Maryland served as a unique tool by which to study host-pathogen interactions including mechanistic hypotheses regarding routes of infection, development of clinical symptomatology and evolution of host immune responses.

Initial improvements in sanitation infrastructure occurring during the last century in "western settings" and subsequently in the rest of the world resulted in a general reduction in the prevalence of typhoid fever. Infection is still highly prevalent in resource-limited countries and travelers. Reasons for this include the lack of effective vaccine campaigns, availability of accurate diagnostic tests and the emergence of antibiotic resistance, hindered by the incomplete understanding of bacterial pathogenesis and response to infection by the native host.

To address this, in 2011 we re-established controlled human infection studies of *S.* Typhi in Oxford. A model was developed using a sodium bicarbonate buffer to neutralize gastric acid and increase bacterial survival through the stomach; neutralization of stomach acid allows the use of lower challenge inocula and a smoother pattern of clinical infection (Waddington et al., 2014b). Ingestion with buffer resulted in a consistent pattern of typhoid infection with an attack rate of 65%, which developed after ingestion of $1–5\times10^4$ colony forming units. Participants were managed after challenge on an outpatient basis.

The development of this new challenge model will provide a standardized approach to study typhoid infection that will prove fundamental for the investigation of immunobiology in the relevant human host and the discovery, development and evaluation of novel vaccines, diagnostics and treatment modalities.

TYPHOID FEVER, A GLOBAL PROBLEM

Typhoid fever is an important health problem in resource-limited settings, while its profile on the world stage is increasing due to the risk of infection for travelers (Leder et al., 2013). Approximately 80% of typhoid fever in Europe and North America is associated with travel, with the greatest proportion resulting from travel to the Indian subcontinent (Hendel-Paterson and Swanson, 2011). Vaccination is recommended by the World Health Organization (WHO) for all travelers to countries where enteric fever is endemic, regardless of the planned duration of stay in a typhoid-endemic setting. Although licensed vaccines provide protection against *S.* Typhi and the live oral vaccine Ty21a is thought to offer limited cross-protection against *S.* Paratyphi B infection, there is currently no licensed bivalent vaccine for *S.* Typhi and *S.* Paratyphi A, the leading causes of enteric fever (Pakkanen et al., 2012). Insights into the dynamics of host-pathogen interactions are crucial to understanding how *S.* Typhi exploits host defenses during infection and is able to occupy its human-restricted niche. Challenge studies could be described as the ideal at-risk travelers model, where naive adult participants are given a bacterial inoculum sufficient to cause typhoid fever. The model can be used to assess the potential of prophylactic vaccination to prevent infection using typhoid challenge and to accelerate the route to licensure, and field testing potential of innovative diagnostics.

Increasing antimicrobial resistance and the evolution of multi drug resistant strains reduces treatment options in many endemic settings (Dutta et al., 2014; Walters et al., 2014). Chloramphenicol, ampicillin and co-trimoxazole resistance emerged throughout the Indian

subcontinent and South East Asia during the late 1980s (Holt et al., 2012). The isolation of quinolone resistant *S.* Typhi has become a cause for concern as drug resistance patterns make treatment options more difficult and costly. Diagnostics at the point of care are important for the prompt detection of causative bacteria, early appropriate case management and targeted antibiotic treatment (Laxminarayan et al., 2013). Large, well designed trials to determine optimal adult and pediatric treatment policies to prevent clinical relapse of enteric fever in endemic areas are important for the management of anti-microbial resistant *S.* Typhi and its associated public health burden (Thaver et al., 2009; Arjyal et al., 2011). In our own challenge programme, there is an opportunity to use the model as a platform to evaluate antibiotic treatment regimens and inform on pharmacodynamics in the resolution of infection.

Prevention of *S.* Typhi infection among travelers through pre-travel vaccination is fundamental as international travel becomes more accessible and a significant cause of travel-associated illness (Freedman et al., 2014). Government and public health authorities are becoming more involved in promoting immunization, personal hygiene education as well as implementing GeoSentinal sites to collect surveillance data including patient demographic characteristics, detailed travel history, vaccination status, and specific typhoid fever disease diagnosis (Leder et al., 2013). Practical applications for human challenge models to characterize infection rate, symptom severity, duration of shedding and biomarkers of disease are important for the identification of disease and mechanisms of protective immunity. Furthermore, the model may provide important supportive data for licensure of new travel vaccines for enteric fever.

WHY ARE HUMAN CHALLENGE STUDIES SO UNIQUE AND WHAT CAN WE LEARN?

Most human *Salmonella* infections result in gastroenteritis and are caused by *Salmonella* Typhimurium or *Salmonella* Enteritidis. *S.* Typhi, the predominant etiologic agent of typhoid (enteric) fever, is specifically adapted to only infect humans with no known animal or environmental reservoir. Animal models for invasive non-typhoidal *Salmonella* (NTS) are available, an important source of human disease in the immunocompromised host (Simon et al., 2011). Despite the discovery and establishment of systemic (intravenous or intraperitoneal) and oral *S.* Typhi murine infection models in humanized and knockout mice (Levine et al., 2001; Andrews-Polymenis et al., 2010; Song et al., 2010; Mathur et al., 2012), the limited understanding of human infection has significantly hampered the development of new vaccines. The availability of tractable animal models of *S.* Typhi infection is of significant scientific interest but it must be argued that improved understanding and assessment of *S.* Typhi host-pathogen interactions can only effectively be achieved through the study of its natural host. The *S.* Typhi human challenge model is pivotal in addressing broad themes of basic *Salmonella* infection.

Human challenge studies involve the administration of *S.* Typhi to consenting healthy adult volunteers with the intent to deliberately induce infection under carefully controlled conditions. Such models are safe and provide a cost effective method to expedite vaccine development and facilitate the discovery and assessment of novel diagnostics. In comparison to large field trials designed to demonstrate vaccine efficacy, challenge models are more cost effective, less labor intensive and can be completed within a shorter study period. Typhoid challenge studies using a relevant host can be extremely informative and have historically contributed to knowledge and understanding of strain virulence, infectious dose, microbial pathogenesis, immunity and the identification of potential vaccine candidates (Hornick et al., 1970a,b, 2007). The re-establishment in Oxford of a controlled human infection model has provided a rare opportunity to make direct comparisons to the findings from historical human studies by using the same challenge strain (Quailes) (Waddington et al., 2014b). The models provide clues to possible correlates of protection, and allow direct comparisons between individuals who do or do not succumb to infection. The collection of baseline (pre-exposure) samples for assessment in combination with pre/post-vaccination and subsequent challenge is a remarkable opportunity. The ability to control the timing of infection in a well-defined study cohort is of unique value and an important research approach by which to understand disease pathogenesis, now greatly enhanced by advances in scientific areas such as functional genomics and systems biology approaches. In addition, identification of host genetic factors linked to infection susceptibility will supplement our knowledge as genetic variation within distinct populations has been linked to defense against typhoid fever (Ziakas et al., 2013).

Despite providing the opportunity for study of *S.* Typhi infection in the natural host, human challenge studies are associated with known limitations. Often only a short-term duration of infection can be studied due to early treatment and patient safety, while the criteria for diagnosis of typhoid fever in the model may differ from that used in an endemic setting. Specific details of the challenge model (including the choice of microbial strain, method of administration and the challenge dose) may not extrapolate to the natural course or mode of infection. Challenge volunteers may not represent the final target population of the vaccine and observations may not always be transferable to an endemic population due to inherent differences in nutrition, host microbiome, or the absence of co-infection with other intestinal bacteria or parasites (Ahmer and Gunn, 2011; Hallstrom and McCormick, 2011; Nuccio and Bäumler, 2014). As the safety of consenting individuals is of the upmost importance, the design of such studies must be ethically sound, ensuring minimal risk to participants while maintaining scientific integrity (Miller and Grady, 2001; Hope and McMillan, 2004).

DEVELOPMENT OF IMPROVED DIAGNOSTICS

Diagnosis of enteric fever in an endemic setting is still heavily reliant on clinical presentation and notoriously unreliable tests reiterate the poor sensitivity and specificity of current diagnostics (Parry et al., 2011). The next generation diagnostic point of care test for *S.* Typhi must be rapid, accurate and affordable in the setting in which it is ultimately destined, and ideally will not rely on expensive equipment, nor highly skilled and

trained clinical and laboratory personnel (Andrews et al., 2013). The search for an improved gold standard typhoid fever diagnostic remains a challenge for all researchers, but one approach is to use samples collected during challenge models as an alternative to those from endemic-settings (Baker et al., 2010; Nga et al., 2010). The model provides a defined time point at which infection takes place and allows the collection of samples before, during and after the onset of clinical disease. Longitudinal challenge model samples (including serum, plasma, saliva, urine, stool and peripheral blood lymphocytes) collected in Oxford will be used to support, validate or indeed refute the accuracy and suitability of a novel diagnostic test.

Recent advances in *Salmonella* diagnostics include metabolomics for the detection of *Salmonella* in blood during infection. Relying on the identification of a unique metabolomic signature in an infected individual, this technique has the potential to discriminate between *S.* Typhi and *S.* Paratyphi A infection (Näsström et al., 2014). Antibody microarrays are being used to identify potential biomarkers of infection for application in the development of novel diagnostic tests and subunit vaccine targets (Liang et al., 2013; Bhuiyan et al., 2014). These approaches are being applied to the challenge model to validate their use at different stages of infection in a naive population. Simple antibody profiles in a small blood sample collected upon hospital presentation have been proposed as an achievable typhoid/paratyphoid fever diagnostic (TPTest) in an endemic setting equipped with a basic microbiology laboratory (Khanam et al., 2013). Using only a few micro liters of a clinical specimen, it is now possible to employ high throughput screening techniques to identify early signatures of the host response, thereby increasing the possibility of a future diagnostic based on a non-invasive sample type such as saliva or urine (Zaka-ur-Rab et al., 2012; Das et al., 2013). Challenge studies provide a unique myriad of resources which through discussion and development with international collaborators has the potential to improve both the quality and standardization of future *Salmonella* diagnostics.

CONCLUDING THOUGHTS

The knowledge gained from human challenge models will not only identify the elusive correlate of protection, but will inform and facilitate the accelerated development of novel diagnostics, identification of novel vaccine candidates, and the targeted assessment of next generation bivalent or combination vaccines. Human challenge models will continue to be instrumental in unraveling the complex pathways associated with infection and will help address remaining questions about *S.* Typhi pathogenesis. We acknowledge the volunteers who participate in *Salmonella* challenge trials and help in the quest to conquer enteric fever.

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