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Immunity to salmonellosis

Gordon Dougan
Victoria John
Sophie Palmer
Pietro Mastroeni

Authors' addresses
Gordon Dougan[1], *Victoria John*[1], *Sophie Palmer*[1], *Pietro Mastroeni*[2]
[1]Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Cambridge, UK.
[2]Department of Veterinary Medicine, University of Cambridge, Cambridge, UK.

Correspondence to:
Gordon Dougan
Wellcome Trust Sanger Institute
Wellcome Trust Genome Campus
Hinxton
Cambridge CB10 1SA,
UK
Tel.: +44 1223 495 391
Fax: +44 1223 494 919
e-mail: gd1@sanger.ac.uk

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Summary: *Salmonella enterica* is a genetically broad species harboring isolates that display considerable antigenic heterogeneity and significant differences in virulence potential. *Salmonella* generally exhibit an invasive potential and they can survive for extended periods within cells of the immune system. They cause acute or chronic infections that can be local (e.g. gastroenteritis) or systemic (e.g. typhoid). *In vivo Salmonella* infections are complex with multiple arms of the immune system being engaged. Both humoral and cellular responses can be detected and characterized, but full protective immunity is not always induced, even following natural infection. The murine model has proven to be a fertile ground for exploring immune mechanisms and observations in the mouse have often, although not always, correlated with those in other infectable species, including humans. Host genetic studies have identified a number of mammalian genes that are central to controlling infection, operating both in innate and acquired immune pathways. Vaccines, both oral and parenteral, are available or under development, and these have been used with some success to explore immunity in both model systems and clinically in humans.

Keywords: *pathogen, macrophage, Salmonella, vaccine, intracellular, immunity*

Introduction

Salmonella are enteric bacteria that are a major cause of infectious diseases throughout the world. These bacteria infect both humans and other animals and are a common cause of zoonotic disease. The genus *Salmonella* incorporates Gram-negative, facultative anaerobic rod-shaped bacilli that are classified as members of the family Enterobacteriaceae. This genus, which is estimated to have diverged from *Escherichia coli* approximately 100–150 million years ago, is genetically diverse and has adapted to colonize many different niches and hosts. For example, *Salmonella* bacteria can be found both as commensal and pathogen in a range of warm and cold-blooded animals and they are capable of surviving free in the environment for extended periods of time. In developed countries, presently *Salmonella* is more commonly associated with acute, gut-associated, non-systemic gastroenteritis; however, specific serovars of *Salmonella* (e.g. *typhi*, *paratyphi* etc.) are historically important as causative agents of the human systemic infection typhoid fever (enteric fever) that is still a common illness in many developing countries. The diversity

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of *Salmonella* is an important consideration when reviewing immunity, as individual bacterial isolates have the potential to differ significantly in terms of antigenic composition, host preference, and virulence potential. Consequently, studies in model systems will be significantly dependent on the particular *Salmonella* isolate under study. The microbiology should not be ignored by the immunologist and visa versa!

A brief word on *Salmonella* classification and nomenclature

Before reviewing the immunology of *Salmonella*-associated infections, it is important to briefly consider aspects of their basic microbiology. The genus *Salmonella* includes two species that infect humans, *Salmonella enterica* and *S. bongori*, each of which harbors multiple serotypes. *S. bongori* is largely restricted to cold-blooded animals and is regarded as an opportunist that will not be discussed further here (1). *S. enterica* is divided into six subspecies, differentiated by biochemical and antigenic characteristics as well as genome phylogeny. The majority of the currently classified human disease isolates fall into *S. enterica* subspecies I. Kauffmann (2) proposed a classification scheme involving further allocation of isolates to serotypes or serovars, based on the serological identification of O [somatic/lipopolysaccharide (LPS)], H (flagella), and K (capsular) antigens. Serovars themselves are not now regarded as separate species, and the nomenclature of *Salmonella* is somewhat confused. Here, for consistency, we italicize the *Salmonella* species name and not the serovar (*S. enterica*, *Salmonella typhimurium*). Serovars in subspecies I include well known pathogens such as *S. typhi*, *S. enteritidis*, and *S. typhimurium*, some of which are frequently used experimentally. For immunologists, the important point about the Kaufmann classification system is that it is based on diversity in antigens found at the surface of the pathogen, possibly indicating diversity driven by immune selection.

Infection syndromes caused by *Salmonella*

Infection by *Salmonella* serotypes can result in varied clinical syndromes or disease states. Indeed, infection by the same isolate can cause clinically distinct disease manifestation in different hosts. The outcome of an interaction between particular *Salmonella* and a potential host is dependent on many factors including serovar, host species, infecting dose, immunologic competence, and gut flora. The spectrum of disease ranges from asymptomatic carriage to a potentially fatal systemic febrile illness. *Salmonella* are generally described as causing either Typhoidal (systemic, febrile) or non-Typhoidal (gut-associated gastroenteritis) salmonellosis (NTS), but this arbitrary distinction breaks down in particular hosts. For example, NTS have recently been implicated as a major cause of invasive bacterial disease of humans in many parts of sub-Saharan Africa, including human immunodeficiency virus (HIV)-infected adults and compromised children (3, 4). This invasive NTS syndrome is currently not common in other parts of the world, including those where HIV infection is relatively common, and it may be associated with particular genotypes of *Salmonella* (5). Finally on this topic, it is worth noting that *Salmonella* can cause other types of infections, including meningitis and osteomyelitis, and *Salmonella* infections have been implicated in arthritis.

Salmonella pathogenicity

Salmonella can infect a wide range of host species, leading to different disease syndromes. However, isolates belonging to some serovars are restricted in terms of the hosts they infect. For example, *S. typhi* only causes typhoid-like disease in humans and some other primates and does not infect normal mice or any other animals to any practical level. Consequently, there is no direct animal model suitable for studying the organism other than exploiting volunteers, although several are under development based on modified mice (6). There are other serovars, such as *S. paratyphi* A, which are also human restricted and cause typhoid-like disease. Isolates of further serovars are host restricted to other animals, such as *S. gallinarum* that causes typhoid-like disease in chickens (7). However, many NTS serovars harbor isolates that are capable of infecting different animals and such isolates are consequently referred to as promiscuous. Indeed, some can cause diseases in animals and spread to humans as zoonotic infections. Importantly, isolates of some of the more promiscuous serovars, such as *S. typhimurium*, can cause an invasive and systemic typhoid-like disease in susceptible mice (8). Consequently, the murine *Typhimurium* model has been exploited as a surrogate for typhoid and general salmonellosis with some success, by microbiologists, geneticists, and immunologists.

An over arching characteristic of *Salmonella* is that they are able to invade and persist in mammalian cells being well adapted to an intracellular lifestyle (9, 10). This includes the ability to colonize macrophages and other immune cells. Indeed, this property has been genetically linked to virulence as mutants unable to survive in such cells generally have a reduced or no ability to cause infection (11). The genomes of *Salmonella* harbor approximately 4500 genes, and over 100 of these genes have been implicated in virulence in some way or

other (12, 13). Virulence-associated genes have been linked to horizontally acquired regions of DNA, but some also map to core regions of the genome shared with other microbes. A number of regions or genetic loci harboring multiple virulence or pathogenicity genes have been defined as 'Pathogenicity Islands'. Two of these, Salmonella Pathogenicity Island I (SPI-1) and SPI-2, encode Type III secretion systems key to the invasive and persistence phenotype (14, 15). For the immunologist, it is important to note that Type III secretion systems, including SPI-1 and SPI-2, can encode needle-like complexes that can 'inject' bacterial proteins directly into host cells. Such injected proteins, often referred to as effector proteins, can hijack host cell functions, including those associated with the immune system. Hence, *Salmonella* are adept at remodeling the host cells they target as well as promoting immunomodulatory activity.

Although SPI-1 and SPI-2 are central to *Salmonella* pathogenesis, many other classes of genes, for example those involved in metabolism or biosynthesis, are also required (16). Thus, virulence *per se* is dependent on the interplay between multiple genes and networks, which can differ significantly between serovars and even individual isolates within a serovar. During infection the bacterium may be starved of nutrients, including essential amino acids that are in short supply in host tissues. Consequently, *Salmonella* auxotrophic for these limiting metabolites may be attenuated in terms of their ability to cause infection (17–19). Such attenuated strains are frequently exploited to extend the period of bacterial persistence in susceptible mice or even as live oral or parenteral vaccines (20). Thus, genetically modified *Salmonella* can be used to explore innate and acquired immunity in both the acute and chronic state of infection. This, together with their ability to target and invade cells, is in part what makes them such attractive tools for immunologists.

The biology of *Salmonella* infection

To understand the molecular basis of *Salmonella* infection, including the ability to colonize and survive within a host, it is important to understand the macro-biologic stages of infection. Much of our current understanding of *Salmonella* infection and the data used to formulate our ideas about mechanisms, are derived from studies using the murine typhoid model. Studies in the mouse have been complemented by the exploitation of other infection models including cattle and birds as well as clinical observations on human disease (21–23).

In natural infection, *Salmonella* are typically acquired from the environment by oral ingestion of contaminated water/food or by contact with a carrier. Interestingly, the environmental reservoir often remains unknown. *S. typhi* is particularly difficult to culture from the environment and tracking outbreaks is challenging. Following ingestion in sufficient numbers, a proportion of the inoculum survives the low pH environment of the stomach to enter the small intestine where infection can be established. Conditions that increase the pH of the stomach can decrease the infective dose. However, *Salmonella* do have an adaptive acid tolerance response, which may aid survival in this environment (24). After passing through the stomach luminal colonization of both the small and large intestines can follow and bacteria can be shed for significant periods. Indeed, *Salmonella* encodes specific proteins such as ShdA that enhance the persistence of colonization (25). Interestingly, some persistence genes are mutated (pseudogenes) in Typhi and Paratyphi A, which have evolved a systemic rather than a luminal lifestyle (12). Many commensal bacteria persist in the lumen of the intestine without obviously interacting significantly with the epithelia or deeper tissues. However, *Salmonella* are predominantly regarded as 'invasive' bacteria and they encode multiple systems (including SPI-1) for interacting with and penetrating the mucosal epithelia. Consequently, to effectively gain access to the epithelium they must avoid neutralizing effects of the immune system, including anti-microbial peptides, immunoglobulin A (26), as well as chemical barriers such as bile salts. Efficient adhesion to the epithelial layer is a prerequisite for invasion (27), and adherence to the apical membrane surface of epithelial cells (enterocytes) is mediated by adhesins such as fimbriae (27). *Salmonella* can mediate direct invasion of the mucosal epithelia; however, at this point, there are several possible routes towards mucosal penetration and systemic invasion. In reality, we know little about how *Salmonella* penetrates the mucosa in man and we thus have to some extent extrapolate from model systems. There is evidence that some *Salmonella* have a preference to exploit the microfold (M) cells, which are specialized epithelial cells that sample the antigenic content of the gut (28–30). *Salmonella* may be able to target M cells and manipulate their function, for example by induce apoptosis (31–33). After moving through M cells *Salmonella* can subsequently access lymphoid cells in tissues such as the Peyer's patches. Within the gut-associated lymphoid tissues (GALT), *Salmonella* can be taken up by cells including those that express CD11c and other dendritic cell markers (34–42). In these early stages of invasion, there is evidence in the mouse that some *Salmonella* may target particular dendritic cell subsets or even locations within the lymph node tissues, for example T-cell rich regions (43, 44). However, the specificities of

cell interactions are likely to vary significantly between isolates and serovars. In an alternative penetration strategy, the *Salmonella* bacterium may be phagocytosed by a CD18-positive cell, which reaches through the epithelial barrier and pulls the bacteria down into the sub-epithelial layers (45–47). Another mechanism may involve the ability of *Salmonella* to disrupt tight junctions, thus deposing the epithelial layer's capacity to moderate ionic balance and immune cell localization (48). In reality, it is likely that a combination of routes is involved.

Relatively little is known about the mechanisms that prevent most *Salmonella* serotypes from becoming systemic at clinically significant levels. Clearly active T-cell immunity is one requirement, as HIV-positive and other immunocompromised individuals are more susceptible to bacteremia caused by NTS isolates (49–51). However, it is likely that the general lack of a strong polymorphonuclear cell (PMN) influx in typhoid facilitates systemic spread through some stealth mechanisms involving a specialized and prepared intracellular location. In typhoid, systemic infection therefore requires a combination of an efficient ability to cross the epithelial layer of the gut, combined with the ability to remaining relatively undetected by the immune system (52, 53). Recent evidence gleaned from genome sequencing *S. typhi* representative of the phylogenic tree of this pathogen suggested that there is almost no obviously selected genetic variation within protein genes (54). Thus, there is no evidence of obvious antigenic variation or immune selection even in known secreted proteins. This would provide further support to the notion that *S. typhi* occupies an immune privileged site *in vivo*. There is complementary evidence that the Vi capsule of *S. typhi* is anti-inflammatory, possibly masking access to pattern recognition molecules by innate receptors (55, 56). Typhoidal *Salmonella* are phagocytosed surreptitiously by macrophages and disseminate through the reticuloendothelial system. In contrast, NTS normally induce a more localized inflammatory response in immune competent individuals, provoking a large influx of polymorphonuclear leukocytes, including neutrophils, to the intestinal lumen and diarrhea (57, 58). Clearly real infections proceed as a complex of interactions between the pathogen and the host, but the above description provides a useful overview of how the initial stages of *Salmonella* infection occur and where early interactions with the immune system can be expected.

To infect systemically, *Salmonella* undergoes either passive or active macropinocytosis to gain entry to targeted cells, including those of the dendritic and macrophage lineage. Once within such cells, they can subvert the normal maturation of the phagosome to form a *Salmonella* containing vacuole (SCV) permissive for survival, persistence and eventually replication (37, 59, 60). In the murine model, *Salmonella* mutants that are defective in the mechanisms required for survival within macrophages are avirulent in mice (11, 61–63). Macrophages that are deficient in natural resistance associated macrophage protein 1 (Nramp-1 or *Slc11a1*) are extremely susceptible to *Salmonella* infection and cannot control replication as efficiently as wildtype macrophages (64, 65). The expression of a functional Nramp1 protein appears to be a key factor contributing to the efficiency by which macrophages restrain the division of *S. typhimurium in vivo* (66). The gene encoding Nramp-1/*Slc11a1* has been reported to encode a proton/divalent-cation anti-porter which is localized to the vacuolar membrane. However, it is worth noting that a genetic association study on typhoid failed to link known *Nramp* human genetic polymorphisms to typhoid disease (67). These data combined with the observation that *Salmonella* are typically isolated from the lymphatic tissues and RES organs during infection, support the hypothesis that survival within macrophages is essential for efficient systemic infection. Following *Salmonella* invasion, the bacterium adaptive responses may sense this phagosomal milieu, low pH and low magnesium ion contents, and consequently *Salmonella* tightly regulate expression of virulence determinants to survive in this endosomal compartment.

Survival and growth in the endosomal compartment are not the only prerequisites for the virulence of *Salmonella*. Continuous spread of the bacteria from infected cells to new infection foci is one of the key features of systemic *Salmonella* infections. This tissue spread avoids high intracellular bacterial densities within the phagosomal compartment, a situation that would render the bacteria either nutritionally or spatially constricted. Bacterial spread from established foci to new infection foci at immunologically unprimed sites is also likely to be a 'hit and run' mechanism that allows *Salmonella* to stay one step ahead of the progressive local activation of the inflammatory response (68–71).

S. typhimurium infection in mice

We emphasized above how studies on inbred mice were used to identify various host factors that contribute to the control of *Salmonella* infection. This early work defined susceptibility in different inbred lineages with some strains deemed susceptible and others resistant (8). Classical genetic studies using a number of mouse lines identified the *Ity/nramp-1/scl11a1* gene described above as the autosomal dominant major trait of innate resistance and also showed linkage of adaptive immunity to the MHC loci (72–75). Interestingly, MHC has also been linked to susceptibility to typhoid in humans (67). Innately susceptible mouse lines have dominated recent

immunology studies in part because of the availability of gene targeted immunodeficient mice on the susceptible background. Virulent *Salmonella* can overwhelm susceptible mice within days even after oral challenge, but the infection can be moderated by using less virulent challenge. Susceptible mice can also be protected against virulent challenge through the use of vaccination, providing a model for monitoring both acquired and protective immunity (76–79). Resistant mouse lines often survive even virulent challenge and a chronic infection can be established that can persist systemically for months (8). This so-called chronic salmonellosis mouse model is currently receiving more interest (65, 80). Susceptibility to salmonellosis can also be influenced by moderating the microbial flora associated with the mouse intestine, for example by using antibiotic treatment (81–83). As new deep sequencing tools become available for defining the composition of the microbiota studies on this previously 'hidden' component are also increasing (84).

Transgenic, knockout, cell transfer experiments, and so-called humanized mice have been used in conjunction with *Salmonella* challenge to explore different aspects of bacterial virulence, host susceptibility, and immunity with great success (85–88). Mutant mice can be challenged and analyzed in depth at different stages of the *Salmonella* infection cycle and these approaches have played a key role in defining aspects of immunity to salmonellosis.

Overview of the *in vivo* immune response to *S. typhimurium*

A schematic representation of infection with *S. typhimurium* in mice is shown in Fig. 1. Several specific *S. typhimurium* isolates such as SL1344, C5 and LT2 are favored in murine studies, as these have been well validated genetically and in terms of their ability to reproducibly infect. Although M cells are thought to be the major portal of entry into the Peyer's patches for *Salmonella* in the mouse, this may not be true in other species. However, in the mouse after penetrating the epithelial barrier, *S. typhimurium* apparently preferentially infect naturally phagocytic cells, such as dendritic cells and/or macrophages, within the *lamina propria* that favor dissemination through the lymphatics and blood to visceral organs: the mesenteric lymph nodes, spleen, and liver (88). *S. typhimurium* harbor a number of mechanisms to invade and persist within both phagocytic and non-phagocytic cells. *Salmonella* are able to survive within the intracellular vacuoles of macrophages by limiting/regulating phago-lysosomal fusion and delaying vacuole acidification. The bacteria that are able to successfully resist killing, for example by the inflammasome response (89–92), begin to grow and replicate intracellularly. During a sub-lethal infection, bacterial growth is controlled a few days postinfection. Conversely, in lethally infected mice, once bacterial titers reach a critical load of approximately 10^8 culturable bacteria per organ, endotoxic shock and rapid death ensue. *Salmonella* disseminate through the lymphatics and blood stream to the mesenteric lymph nodes and to deeper tissues. Eventually invading bacteria are transported to tissues and organs including the spleen, liver, and bone marrow, where they undergo further intracellular replication or potentially lay dormant (Fig. 2).

When considering immunity to salmonellosis, it is worth focusing on what we have learned from the mouse model as this has been worked on most extensively. Working from this

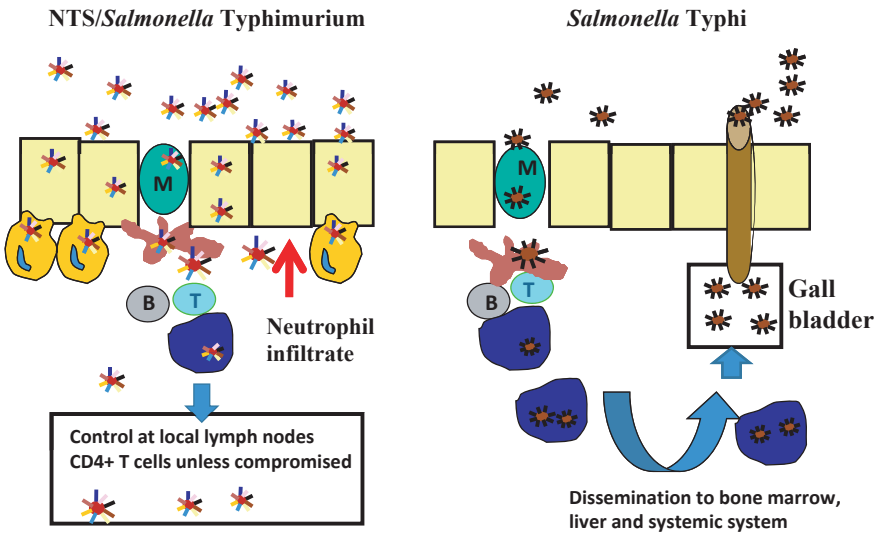

Fig. 1. *Salmonella Typhimurium* infection in mice. Outline route of infection associated with invasive salmonellosis.

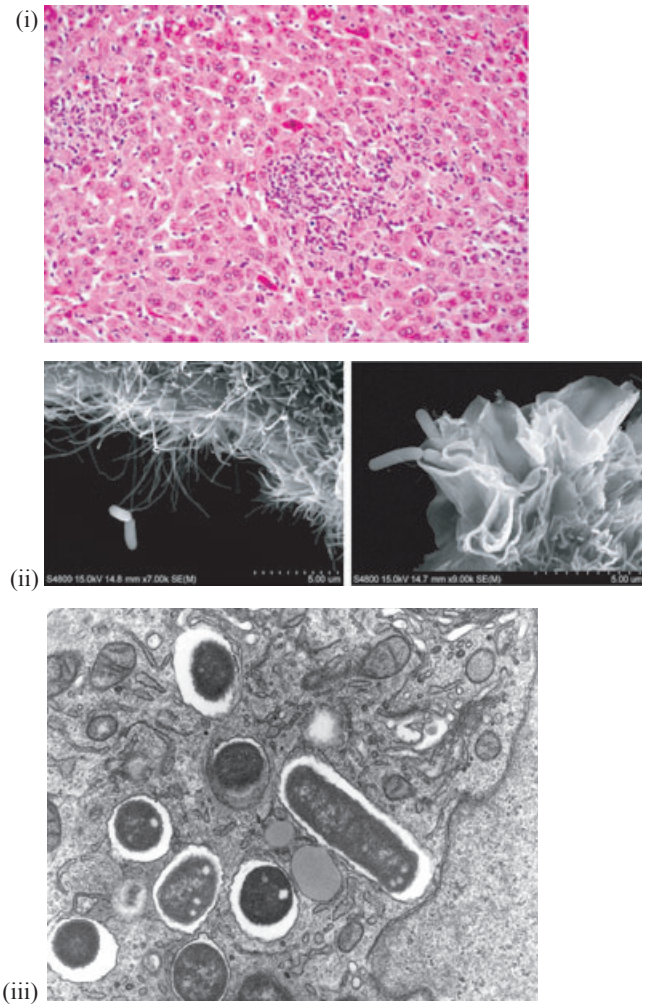

***Fig. 2.* Histochemical and electron microscopic images of Salmonella associated with the host.** (i) A H&E stain showing clear evidence for a *Salmonella* associated granuloma in the liver of an infected mouse. (ii) Scanning electron micrograph of salmonella attaching to a macrophage. (iii) Transmission electron micrograph of *Salmonella* residing within an adapted vacuole within a murine cell.

basis, comparisons can be made with other systems. Mice can be challenged via a number of different routes, including oral, intravenous, and intraperitoneal, and the route of choice will influence subsequent immunity. After oral challenge, *Salmonella* will more efficiently stimulate components of the gut associated immune/lymphoid system. From oral studies, we know secretory IgA can contribute to the control of infection but it does not appear to play a major role and immunity can be mediated in the absence of secretory IgA (93). Defensins and similar anti-microbial peptides also contribute to early control along with other innate factors (94). Whatever route of challenge is employed, in the mouse *Salmonella* bacteria are quickly found both within deeper tissues and within immune cells, particularly macrophages (94). The subsequent growth and survival of *Salmonella* will then differ according to the mouse genetic and immunologic background. In healthy humans, NTS remain predominantly associated with the gut mucosal and GALT, although systemic spread can occur with *S. typhimurium* in compromised individuals. In different hosts such as veterinary species, the outcome differs in terms of local versus systemic spread and again this is influenced and complicated by the properties of the infecting isolate and the specific breed of animal (this topic will not be covered here).

In previous descriptions of the mouse model, the infection has been arbitrarily divided into different phases, partly as a means of simplifying description and analysis. Of course, such phases are actually highly integrated, but this approach will be used here (*Fig.* 3). Phase 1 covers the period between when *Salmonella* actually enter tissues, normally via the surfaces of the gut, but this may actually differ in experimental procedures in the laboratory. During this first phase, bacteria pass through the epithelia and disseminate *via* the efferent lymphatic and circulatory systems to tissues such as the bone marrow, liver, and spleen. In phase 1 both rapid division and bacterial killing occur the latter prevailing and resulting in a significant loss of the challenge inoculum at this stage. This killing phase is partially dependent on the production of reactive oxygen intermediates and is short lasting (63, 71). At this point, serum antibody and their associated effectors are also important in controlling infection by enhancing clearance of the bacteria from the blood and their initial kill. However, in the susceptible mouse, antibody alone does not normally mediate complete protection, because the humoral response, in isolation, is unable to abort the growth of the bacteria in the tissues in the later phases of the infection when an interplay between humoral and cellular responses is key. This is likely to be true in humans, but it is worth noting that vaccines based on the T-cell-independent antigen, Vi polysaccharide, can provide some (but not high level or long lasting) immunity to typhoid (95–98). Vi conjugate vaccines that potentially draw in T-cell help and thus are likely to trigger a longer lasting, isotypically switched, and affinity matured anti-Vi response have shown initial promise in enhancing Vi-mediated protection (99).

In phase 2, bacteria will undergo a period of replication, predominantly within cells, and killing becomes negligible (71, 100). Clearly a slower net growth rate favors survival in the face of an accumulating acquired immune response, and this phase is thus critical for determining the outcome in terms of morbidity and mortality. The rate of growth within cells is known to be influenced by innate factors such as Nramp-1 and reactive oxygen intermediates, and this is manifest through the graphed slope measuring *in vivo* tissue growth

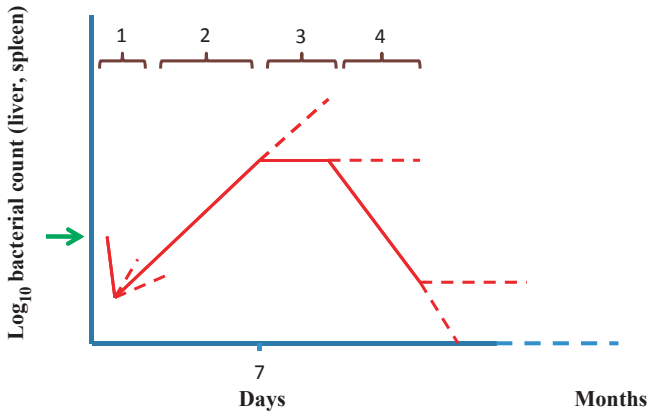

***Fig. 3.* The steps in *Salmonella* infection.** In this review we have arbitrarily divided *Salmonella* infection into a series of stages. These are illustrated in this figure. In reality the stages can overlap significantly in time and space in different infections. Salmonellosis is essentially an invasive infection, although bacteria do exist free in the lumen of the intestine in significant numbers, particularly in non-Typhoidal salmonellosis infections. After oral challenge, *Salmonella* will encounter physical, chemical, and other innate challenges which can mediate significant control. Upon contacting the luminal epithelia *Salmonella* bacteria will target specific entry routes in order to transverse to deeper tissues. This can involve various cell types. Once within tissues, the *Salmonella* will be exposed to the immune system. We believe that there is both an extracellular and intracellular phase within minutes and hours of penetration (Phase 1). The extracellular bacteria will be targeted by antibody and complement (and other humoral factors). The intracellular bacteria can be killed by phagocytes before they can adapt to intracellular life. Neutrophils are important in gastroenteritis. Phase I is often manifested as an initial kill, where bacterial numbers quickly fall. In phase II, *Salmonella* are largely intracellular and are located within an adapted *Salmonella* vacuole, where they can resist killing, Here in the naive animal, Nramp and cytokines such as IFNγ, TNFα, and Th-associated factors play a critical role in controlling infection and the rate of growth. Granuloma formation is critical. The Th17 response is associated with controlling local gut associated infection. In phase III, acquired immunity kicks in to further control and start to clear infection. Here, $CD4^+$ T cells are critical with $CD8^+$ and other T cells playing a lesser role. Again IFNγ and TNFα are important cytokines.

over time (Fig. 3). Where the balance of bacterial virulence and host resistance state favors the pathogen, bacterial growth continues rapidly until *Salmonella* accumulate to levels where death is inevitable and no obvious slowing of the net growth rate occurs. In innately resistant mice, in mice infected with *Salmonella* of moderated virulence, or in animals with primed acquired immunity (or any combination), the growth rate in phase 2 is slow and this allows the gradual onset of a response that suppresses the infection and bacterial numbers reach a plateauing phase 3. This normally becomes apparent towards the end of the first week after challenge, with the plateau in bacterial numbers lasting approximately a further 1–2 weeks. During the final or fourth phase, bacterial numbers in the liver and spleen begin to fall progressively until complete clearance is achieved. In some mice, for example those bearing some H-2 haplotypes, this plateau phase can actually last for months and in some cases the infection is never cleared.

The innate immune response to *S. typhimurium*

During the early phases of infection, the production of a number of cytokines and soluble factors as well as the recruitment of bone marrow derived macrophages and the development of organized granulomas have been shown to be critical for controlling *Salmonella* spread and growth in the reticuloendothelial system. Macrophages and neutrophilic granulocytes are decisive for controlling the net growth of bacteria during the early phase, and they exert a bactericidal activity in the first few hours of the infection that is replaced later by bacteriostatic functions (71).

The formation of multicellular pathological lesions at the foci of infection is critical for the effective control of *Salmonella*, particularly in tissues such as the spleen, lymph nodes and liver (101). Suppression of bacterial growth in the reticuloendothelial system coincided with the formation of macrophage rich multicellular lesions and the administration of anti-TNF-α antibodies exacerbated the course of a *Salmonella* infection in both susceptible and resistant mouse strains by inhibiting their formation (101–105). In mice genetically lacking TNFα, these granulomas are not formed efficiently, and the *Salmonella* can be found widely distributed within the liver tissues surrounded by increasing pathological signatures as the infection rapidly worsens (106). Treatment with anti-TNF-α antibodies well after the suppression of bacterial growth, and formation of granulomatous lesions can prompt a relapse of the infection and a regression of already established granulomas suggesting that TNF-α is required throughout infection (104).

Fluorescence microscopy studies and novel genetic tagging approaches, where individual clones of replicating *Salmonella* can be identified, have dissected the patterns of the formation of individual infection foci and subsequent bacterial escape (71). Such studies have clarified how the infection develops at a local level, have highlighted the dispersive nature of the infection process, and have indicated escape from granulomas as a key mechanism in *Salmonella* virulence.

Components of the bacterial cell wall such as LPS, DNA, flagella, and certain lipoproteins activate TLRs on host cells, which in turn induces a robust inflammatory response within tissues, characterized by the production of Th1-like cytokines such as IFN-γ, TNF-α, and IL-1, IL-6, IL-12, and IL-18, as well as macrophage migration inhibitory factor and iNOS (86, 107–110). IFN-γ has been shown to be vital for resistance against infections involving intracellular pathogens,

including *S. typhimurium*, in part because of stimulation of the antibacterial activity of macrophages. Mice treated with anti-IFN-γ antibodies or gene-targeted mice lacking the IFNγR are impaired in their ability to clear a sub-lethal dose of virulent *S. typhimurium*, and they eventually succumb to infection (79, 111). IL-12 and IL-18, secreted by activated macrophages, act both independently and synergistically on natural killer cells and helper T cells to induce the production of further IFN-γ, which activates the macrophages through a positive feedback loop (86, 108). Additionally, IL-12 is important for the polarization of T-helper cells toward the Th1 response (112–114).

The adaptive antigen-specific immune response to *S. typhimurium*

T cells do not play a key role during the early phases of infection control in naive animals, as nude, αβ T-cell knockout, as well as $CD4^+$ or $CD8^+$ T-cell-depleted mice are all capable of suppressing the early growth of *Salmonella* (115). Despite the lack of an apparent role early in infection, studies in the mouse have suggested that T cells may be activated soon after oral and parenteral *Salmonella* infection (116). $CCR6^+$ dendritic cells in the Peyer's patches may be involved in local T-cell activation after oral challenge and B-cells are also implicates in T-cell activation (see below) (40, 117).

However, while the innate immune response is highly successful in controlling the initial growth of *S. typhimurium*, it is insufficient for achieving full protective immunity. Effective control and eventual eradication of bacteria during the late phases of a primary infection and the generation of protective immunity against subsequent infections requires the development of a *Salmonella*-specific T-lymphocyte response and the active recruitment of such cells to the site of infection (115).

In primary infections, $CD4^+$ $\alpha\beta TCR^+$ T cells with a Th1 phenotype mediate the clearance of the bacteria from the tissues with little or no obvious contribution of $CD8^+$ T cells (115). B cells and antibodies are also largely dispensable in the late stages of primary salmonellosis. αβ T cells appear to be more important than γδ T cells since mice on a susceptible background and deficient in γδ T cells are able to control systemic infection with an attenuated strain of *S. typhimurium* while mice containing defects in the αβ T-cell receptor are not. During a primary infection, T cells also mediate the regulation of *Salmonella*-specific B-cell activation and maturation, which results in the production of isotype switched antibodies against bacterial polysaccharide and protein antigens (118). Contrary to what is seen in primary infections, resistance to virulent challenge in innately susceptible vaccinated mice requires the concerted action of both $CD4^+$ and $CD8^+$ T-cells with the additional requirement for anti-*Salmonella* antibodies (101, 119). It is likely that the T-cell response confers protection via activation of the mononuclear cells where *Salmonella* resides, while antibody targets the bacteria that escape from infected cells and travel, via the extracellular space, to distant sites in the tissues to establish new foci of infection. In this context, $CD4^+$ cells probably mediate protection via the production of cytokines, in particular IFN-γ, and through IFN-γ-independent mechanisms including the production of other macrophage-activating cytokines (120–122). In fact, the depletion of Th1-like cytokines such as IFN-γ, TNF-α, and IL-12, using neutralizing antibodies after vaccination, greatly exacerbates secondary infection (86, 101). Studies with defined T-cell populations have reinforced the critical role of Th1 and IFNγ in controlling salmonellosis in murine models (79). $CD8^+$ T cells differentiate into cytotoxic T lymphocytes, which may also play a role in protection by liberating intracellular *S. typhimurium* from infected macrophages. Thus, the activation of Th1 cells is required not only for the defense against primary infection with *Salmonella* but also for the vaccine-induced resolution of infection.

There has been a great deal of recent interest in the role of Th17-producing T cells in the control of *Salmonella* infections. HIV has highlighted the critical role T cells play in controlling systemic spread, at least with NTS bacteria (52, 123, 124). Th17 cells are now defined as a distinct lineage to Th1 and Th2 cells and are characterized by the expression of cytokines such as IL17A and F, IL-22, and IL-26. The distribution of the receptors for some of these cytokines has highlighted the role of epithelia and local T cells in *Salmonella* control. IL-17 and IL-22 are induced during some *Salmonella* infections and recent work in mice, calves, and the macaque has suggested that these cells are critical for controlling local invasion by *Salmonella*. For example, macaques co-infected with SIV are disrupted in this system and exhibit higher levels of systemic bacteria (125). Such work may explain, at least in part, some of the reasons why invasive NTS are now common in Africa, although interactions with malaria may also be very important (126). IL-17 and IL-22 are important for activating and coordinating mucosal immune responses, including the expression of chemokines and certain classes of defensins such as the RgIII class of C-type lectins (123, 125). Host associated lipocalin-2 may also play a role mucosally, sequestering or interfering with bacterial iron savaging systems (127).

Although a great deal of work has gone into trying to identify the *Salmonella* antigens involved in T-cell responses this has met with limited success. Several groups have shown, perhaps

not surprisingly, that flagella are a T-cell target and a number of flagella associated T-cell epitopes have been identified (128, 129). Immunization with flagella can induce partial protection against *Salmonella* challenge in some model systems, although there is a debate as to where anti-flagella T cells immunity might act as flagella expression is highly regulated and may only be at low level when bacteria are resident within cells or even the systemic system (it may also vary between serovars) (130–132). Interactions between bacterial effector proteins and T cells may be involved in delaying the action of T cells but more work is required to define if this is actually the case.

Role of B cells during the immune response against *S. typhimurium*

Resolution of a primary infection in mice with *S. typhimurium* is a combination of innate and T-cell-mediated effects with a less significant role for B cells and antibodies. B cells and antibody can and do contribute to protection against secondary infection in mice and they also play a role in humans (66, 87, 88, 133–135). For example, in vaccinated mice (or in mice that received antibody via passive transfer), antibodies contribute to control in the very early stages of an infection by enhancing bacterial killing before *Salmonella* has reached an intracellular location (136); antibody enhances the uptake of *Salmonella* via FcRI and increases the ROI-mediated anti-microbial functions of phagocytes (137, 138). In humans, antibody has been implicated in early protection in children against invasive NTS in Africa (135).

B cells have been shown to be essential for the expression of full protective immunity to virulent oral challenge. Igh-$6^{-/-}$ mice infected with a live, attenuated *S. typhimurium aroA* vaccine strain were able to control and clear the inoculum from the reticuloendothelial system (66, 87). However, unlike wild-type controls, Igh-$6^{-/-}$ mice challenged orally with virulent *S. typhimurium* 4 months after vaccination, were unable to control infection, suggesting that B cells are required for acquired resistance. Mice defective in the microRNA mir155 or BIC have an impaired B-cell function, and they respond poorly in terms of antibody production against vaccine antigens (139). Interestingly, mir155 knockout mice do not mount an effective protective response to salmonellosis following primary immunization and secondary virulent challenge, further emphasizing the importance of B cells in protective immunity. It appears that the role of B cells involves more than just the production of *Salmonella*-specific antibodies since passive transfer of large amounts of immune serum into immunized Igh-$6^{-/-}$ mice before challenge did not fully restore resistance. B cells contribute significantly to the engenderment and expansion of anti-*Salmonella* Th1 cells. In fact, protective Th1 responses to *S. enterica* do not develop effectively in the absence of B cells. Total splenocytes and purified $CD4^+$ T cells isolated from Igh-$6^{-/-}$ mice after vaccination showed a reduced ability to release the Th1-type cytokines IL-2 and IFN-γ upon re-stimulation *in vitro* with *S. typhimurium* antigens (116). Further work in chimeric mice dissected the early (innate) and late (cognate) contributions of B cells to Th1 programming. B cell-intrinsic MyD88 (myeloid differentiation factor 88) signaling was seen to be required for primary effector Th1 development, whereas antigen specific-specific BCR-mediated antigen presentation is necessary for the development of memory *Salmonella*-specific Th1 populations (140).

Human salmonellosis

It is important to further consider aspects of immunity to salmonellosis in humans. In real terms, we know relatively little about the direct immunology of human *Salmonella* infections, but useful lessons can be obtained by observing the incidence of salmonellosis in patients with immunodeficiencies. Both defects in innate and acquired immunity can predispose humans to salmonellosis, and often for reasons that are unclear, NTS is the most common among the *Salmonella* syndromes to be associated with human immunodeficiency. The knowledge and awareness of those immunologic defects that can predispose humans to *Salmonella* infection has important implications for the understanding of how the immune system controls these infections as well as for the improvement of the immunogenicity and safety of currently available vaccines. In fact, some live attenuated *Salmonella* vaccines such as aromatic dependent (*aroA*) mutants or *htrA* mutants can cause lethal infections in mice deficient in T cells, IL-12, or IFNγ (86, 115, 118). Other vaccines (e.g. SPI-2 mutants of *Salmonella* regain virulence in NADPH oxidase or RNI deficient animals (60, 141). Therefore, some of these vaccines could prove dangerous in patients with immunosuppressive syndromes such as chronic granulomatous disease (CGD) (see below), in individuals with IFNγ or IL-12 defects, and in those with impaired T-cell immunity. These immunodeficiencies may be latent or undiagnosed in the individual at the time of vaccination.

Anatomic and physiologic factors operating in the gastrointestinal system influence the intestinal phase of *Salmonella* infection. For example, conditions that reduce gastric acidity or cause rapid gastric emptying time determine an increased risk

of infection (142). Human defensin 5 (HD5) is an anti-microbial peptide produced by Paneth cells in the crypts of Lieberkuhn. Expression of HD5 in transgenic mice increases resistance against oral *Salmonella* infection and raises the possibility that this defensin may play an important role in human salmonellosis (143, 144).

CGD comprises a group of disorders associated mutations in genes encoding subunits of the NADPH oxidase. These defects render phagocytes incapable of killing ingested microorganisms via oxygen-dependent pathways. CGD patients also develop septicemia due to *Salmonella* and other Gram-negative enteric bacteria (145). An association between *Plasmodium falciparum* malaria and NTS bacteraemia has been identified (50), and the mechanism is attributed to macrophage dysfunction due to the breakdown of hemoglobin that results in the production of hemozoin leading to defective phagocytosis and decreased NADPH oxidase-dependent respiratory burst. NTS is a common causes of septicemia in sickle cell disease (SCD) (146), most likely due to secondary defects in macrophage function.

Patients with immunodeficiency syndromes in antibodies and/or B-cell functions are more susceptible to salmonellosis. Anti-*Salmonella* antibody appears to play a critical role in controlling salmonellosis early in human life and any defect in this response enhances susceptibility (135). Bactericidal activity is critical. Antibodies transferred from immune mothers offers some protection for the early months after birth but a 'susceptibility window' opens until the children develop their own protective antibodies. A functional complement system is also key and anti-*Salmonella* activity can be directed in both an antibody dependent and independent manner (135). Some B-cell immunodeficiencies may lead to incresed susceptibility to *Salmonella* not just due to impairments in antibody responses but also to the disruption in the cross-talk between T-cells and B-cells, known to be essential for acquired resistance in mice. For example, persistent diarrhea due to *Salmonella* was reported in individuals with X-linked agammaglobulinaemia (XLA), which is characterized by a profound deficiency of B-cell development due to mutations of the tyrosine kinase Btk (147). Increased risk of salmonellosis has also been reported in patients with common variable immunodeficiency (CVID) that is characterized by hypogammaglobulinemia and abnormalities of cell-mediated immunity (147, 148). Good's Syndrome is a rare disease characterized by B lymphopenia, hypogammaglobulinemia, and variable defects in cell mediated immunity including $CD4^+$ T lymphopenia and an inverted $CD4^+$:$CD8^+$ T-cell ratio (149, 150). Intestinal pathogens including NTS have been isolated in a few of these patients (149, 150). X-linked hyper IgM syndrome is a rare genetic disorder due to mutations in the gene encoding CD154 (CD40 ligand), which is expressed on activated T cells and is characterized by recurrent infections in association with markedly decreased serum IgG, IgA, and IgE levels but normal or elevated IgM levels. Mutations in CD154 lead to a lack of appropriate interaction between T cells and B cells leading to failure of B-cell isotype switching and memory generation. *Salmonella* infections have been describes in a minority of patients with X-linked hyper IgM syndrome (151).

Cytokines networks play a key role in resistance to salmonellosis in humans due to their ability to activate the anti-microbial functions of macrophages. The polymorphic allele variant TNFA*2 (−308) has been associated with susceptibility to typhoid fever (152). Patients with defects in IL-12 receptor β1 subunit (IL-12R), IL-12 p40 subunit (IL-12), IFNγ receptor chains 1 and 2 (IFNγR), and STAT-1 show increased susceptibility to salmonellosis. In particular, complete IFNγR (c-IFNγR) and complete STAT-1 deficiency result in the most severe infections with a poor outcome underlying the key role of this cytokine in human salmonellsis (153–157). Mononuclear cells from a NTS patient with X-linked anhidrotic ectodermal dysplasia with associated immunodeficiency (EDA-ID), caused by hypomorphic mutations in the gene encoding NF-κB essential modulator (NEMO) (158), showed impaired cellular responses to IL-18 and IL-1β. IL-18 and IL1-β show strong synergy with IL-12 in the induction of IFNγ production, thus providing an explantion for the susceptibility of this patient to salmonellosis.

T cells are also important for protection in humans. For example, HIV infection with its profound suppressive effects on T-cell and macrophage-mediated immunity, results in markedly increased susceptibility to *Salmonella* infection (159, 160) protracted relapsing disease often with associated multivisceral foci of infection and septicemia. The enhanced susceptibility to systemic NTS disease has been linked both to humoral (135) and cellular (123) facets of the immune response, including the Th17 pathway. Antibody itself is not protective when HIV-positive individuals lose $CD4^+$ T-cell counts and display the symptoms of AIDS. Evidence indicates that the circulating antibodies in HIV-positive individuals may actually block the ability of complement to kill *Salmonella* (135, 161). The immunologic basis for this observation is still under investigation.

MHC class II deficiency on the cell surface is a rare disease with around 70 documented cases worldwide and is due to defects in transacting factors (CIITA, RFXANK, RFX5, and RFXAP) that are essential for normal MHC class II gene

expression (162). Lack of MHC-II expression leads to panhypogammaglobulinaemia, reduced specific anti-microbial antibody responses, and severe $CD4^+$ T-cell lymphopenia. Bacterial infections are dominant, and *Salmonella* is among the most common pathogens isolated from these patients. Thus, it appears that MHC class II-dependent antigen presentation and normal $CD4^+$ T-cell function is essential for anti-*Salmonella* immunity in humans.

In the case of typhoid (*S. typhi*, *S. paratyphi*), an acute febrile infection can be complicated by secondary immunologic complications including perforation of the intestine (23, 163). Further, many humans can spontaneously relapse or be reinfected several months or years after recovery, suggesting protective immunity is not complete (164, 165). Anti-Vi antibody can offer protection, but the role of antibodies to other antigens is not well defined in humans. Certainly otherwise apparently healthy individuals can relapse even when they have high titers of circulating anti-Typhi antibodies. Some individuals become persistent carriers and sporadic shedders of *S. typhi* and such individuals are a serious threat to the community (Typhoid Mary as an example). The gallbladder and possible lymphoid sites have been implicated as sites where *S. typhi* can persist. Gallbladder persistence has been associated with stones or other pathological features and Typhi may exist here in the form of specialized communities such as biofilms. The role of carriers in maintaining the human restricted pathogen *S. typhi* in the human population over time is likely to have been critical.

Challenge studies with virulent *S. typhi* were performed throughout the 1950s and into the next decade, but such challenges have not been repeated in recent years. However, human challenges using specifically attenuated *S. typhi* live oral vaccines have continued and this has provided an opportunity for immunologic investigations (166, 167). Oral immunization with live vaccines such as Ty21a can induce circulating anti-Typhi antibodies, and there is some weak correlation with antibody production (using some formulations) and protection (166). However, live oral vaccination can induce both $CD4^+$ and $CD8^+$ T-cell responses, and these have been characterized in some depth. Sensitized lymphocytes capable of expressing Th1 cytokines such as IFNγ, TNFα, and IL-10 (but not IL-4 and IL-5) appear in the peripheral blood in the days following oral immunization with a number of live oral typhoid vaccines. These lymphocytes proliferate and produce IFNγ in response to a number of *S. typhi* antigens including flagella (168). $CD3^+CD4^+CD8^-CD56^-$ T cells are apparently the predominant IFNγ-secreting cells associated with this response. $CD8^+$ cells are also present that secrete IFNγ (169–171). These cells may play a role in enhancing macrophage and antigen presenting responses. $CD8^+$ cells are associated with $CD3^+CD8^+CD4^-$ T cells that can mediate a cytotoxic response to cells infected with *S. typhi* (169–171). Interestingly, a non-classical (HLA-E-restricted) cytotoxic T-cell response has also been detected following immunization with oral Ty21a vaccine (172). These cells can also kill *S. typhi* infected cells. A subset of these circulating cells expresses $\alpha4\beta7$ gut-homing integrin. Thus, gut-associated cells are likely to be important in controlling *S. typhi* dissemination. IgA-secreting B cells are also present in circulation, and such cells have been found to be useful markers for assessing vaccine 'take' through enzyme-linked immunospot assay-type approaches (172).

Concluding remarks

The shear diversity of the species *S. enterica* in terms of antigenic composition and pathogenic potential makes an assessment of mechanisms of immunity *in vivo* extremely challenging. Clearly many different arms of the immune response can be and are engaged in controlling infection in both the naïve and immune host. Mucosal and local immunity is critical in controlling infections by NTS, but when this breaks down, life-threatening systemic infection can follow. With the typhoidal serovars systemic infection and immune evasion is the norm and full protective immunity is rarely displayed even following natural infection. Some of these phenomena can be studied in the mouse, but although the murine system is useful for exploring mechanisms, observations do not always correlate with human immunity. More clinical studies will be required in the future if we are to understand how to design more effective vaccines to control Salmonella disease.

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