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Current Opinion in Immunology

Review

Determinants of persistent *Salmonella* infections

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Persistent bacterial infections constitute an enormous challenge for public health. Amongst infections with other bacteria, infections with typhoidal and nontyphoidal *Salmonella enterica* serovars can result in long-term infections of the human and animal host. Persistent infections that are asymptomatic are difficult to identify and thus can serve as a silent reservoir for transmission. Symptomatic persistent infections are often difficult to treat as they harbor a combination of antibiotic-tolerant and antibiotic-resistant bacteria and boost the spread of genetic antibiotic resistance. In the last couple of years, the field has made some major progress in understanding the role of persisters, their reservoirs as well as their interplay with host factors in persistent *Salmonella* infections.

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Current Opinion in Immunology 2023, **82**:102306

This review comes from a themed issue on **Chronic Infections**

Edited by **Thomas Mertens**

For complete overview of the section, please refer to the article collection, "Chronic Infections (2023)"

Available online 27 March 2023

https://doi.org/10.1016/j.coi.2023.102306

Introduction

Gram-negative *Salmonella* bacteria are ingested with contaminated food or transmitted via the fecal-oral route. Pathogenic serovars cause a broad range of diseases, including enterocolitis, enteric fever, and bacteremia [1]. Infections with typhoidal serovars (TS), including *S.* Typhi and *S.* Paratyphi, are restricted to humans and can lead to a life-threatening, systemic disease called enteric or typhoid/paratyphoid fever [2]. Nontyphoidal *Salmonella* serovars (NTS), including *S.* Typhimurium and *S.* Enteritidis, can infect a broad range of hosts. In immunocompetent adults, NTS usually cause self-limiting gastroenteritis with occasional secondary bacteremia [3]. *Salmonella* infections can result in either acute infections with subsequent clearance of the pathogen, persistent infections or death of the host. Persistent infections can follow asymptomatic or symptomatic acute infections that are not fully cleared by the host immune system resulting in continuous shedding of the bacteria with the feces as a source for transmission. In persistent infections, the pathogen is able to colonize the host for long periods of time. *Salmonella* manipulates the host immune system to its own benefit and can alter its metabolism to withstand antimicrobial drug treatment. *Salmonella* resides intracellularly or extracellularly in the intestinal or gall bladder lumen which may result in persistent or periodic fecal shedding [2,4].

In this review, we highlight the recent progress in our understanding of persistence mechanisms of typhoidal and nontyphoidal *Salmonella enterica* serovars, their sites of persistence as well as the different lifestyles promoting long-term survival and describe the modulation of the immune response facilitating persistent infection.

Persistence rate and duration of shedding

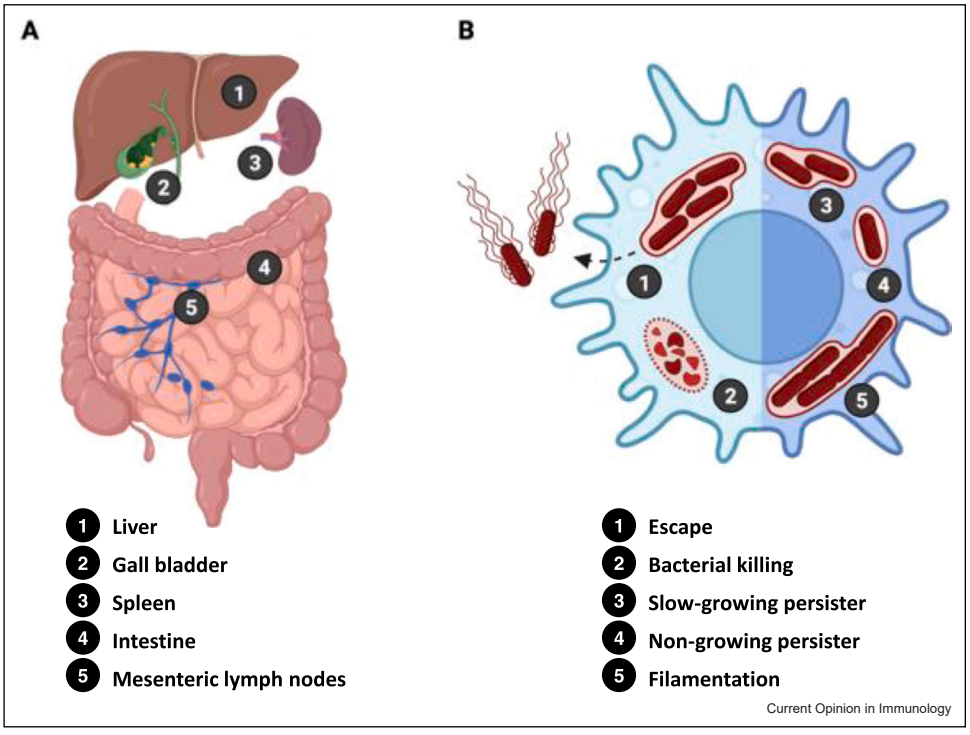
Up to 4% of *S.* Typhi patients fail to eradicate the infection and develop an asymptomatic, chronic carrier state periodically shedding bacteria in their feces for more than one year [5–7]. In contrast, most infections with NTS serovars are cleared within 12 days [8]. However, about 2.2% of NTS patients become persistently infected (defined by culture-confirmed isolation of the same serovar from stool or, in rare cases, also from extra-intestinal sites) with infection durations ranging from 30 days to 8.3 years [9]. In contrast to (often asymptomatic) typhoid carriers, over 65% of NTS patients had symptoms including recurring diarrhea. Infections with NTS serovars result in severe dysbiosis of the gut microbiota which further perpetuates diarrhea by affecting glycan metabolism and short-chain fatty acid synthesis and consequently epithelial barrier functions [10,11].

The frequency of persistence varies between NTS serovars and *S.* Mbandaka, *S.* Bredeney, *S.* Infantis, and *S.* Virchow were most frequently associated with persistent infections [9].

Sites of persistence

Salmonella can occupy different permissive niches to establish persistence (Figure 1A). *Salmonella* located in

Figure 1



Sites of *Salmonella* colonization and persistence. **(A)** *Various body sites can harbor Salmonella during persistent infections:* Macrophages have been shown to be a major cell type for persistence. Persistently infected macrophages have been found in granulomas in lymph nodes (5), liver (1), and spleen (3). These macrophages display an anti-inflammatory M2-like polarization. In addition, the gall bladder (2) can harbor persistent typhoid salmonellae located in epithelial cells and the gall bladder lumen or as biofilms on gallstones. Furthermore, intestinal bacteria (in the lumen and gut wall) (4) are responsible for fecal shedding. Since the lifespan of enterocytes is short, immune cells in the gut mucosa might serve as a survival niche for *Salmonella*. In addition, intestinal persistence facilitates the exchange of resistance plasmids. **(B)** *Salmonella phenotypes found within macrophages:* after the infection of macrophages, *Salmonella* resides inside the *Salmonella*-containing vacuole (SCV) where the bacteria can actively replicate. By inducing macrophage cell death, *Salmonella* escapes and can infect neighboring cells (1). Virulence gene expression is needed to establish the SCV and dampen the host immune response to avoid killing by the phagocyte (2). Adopting different physiological states allows *Salmonella* to combat intracellular stress and antibiotic treatment, but active gene expression is needed to resume growth in more favorable conditions: *Salmonella* can either slow down the growth rate (3), arrest growth (4), or switch to a filamentous phenotype with (transiently) suppressed cell separation (5). The fate of persistently infected macrophages is unclear. Besides macrophages, other cell types, such as DCs and fibroblasts, are discussed as possible niches for *Salmonella* persistence (created with BioRender.com).

the intestinal lumen can breach the intestinal epithelial barrier and subsequently translocate systemically where it can be found in various cell types including epithelial cells (e.g. in the gall bladder), intestinal fibroblasts, and macrophages in the mesenteric lymph nodes (MLNs), spleen and liver.

After oral uptake of contaminated food, *Salmonella* resides in the intestinal lumen which constitutes another possible niche for long-term colonization. *Salmonella* Typhimurium induces and takes advantage of local inflammation to compete with the intestinal microbiota to overcome colonization resistance in the gut and to survive in the altered ecological niche [12,13]. Administration of antibiotics has been demonstrated to prolong persistent NTS infections and the duration of shedding in humans [14,15] and mice by further affecting the microbiota composition and/or by selection for antibiotic persisters [16,17]. The intestinal microbiota does not only play a role in inhibiting the initial colonization of the pathogen but is also important for pathogen clearance [18].

After translocating through the intestinal epithelium, *Salmonella* has to overcome host immune defenses to spread to other body sites by infecting subepithelial phagocytes such as macrophages and dendritic cells (DCs). Subsequently, the bacteria spread within the phagocytes to systemic sites, including the MLNs, spleen, liver, gall bladder, and bone marrow, where they can establish persistence [19].

In *S.* Typhi infections, the gall bladder as well as other parts of the human biliary tract represent the major sites

of persistence. The bacteria can multiply and persist extracellularly in the gall bladder lumen, for example, as biofilm on gallstones or within gall bladder epithelial cells [20]. The presence of gallstones as well as other preexisting biliary diseases facilitates the establishment of *Salmonella* persistence in the biliary tract [21,22]. Although the gall bladder seems to be the primary site of *S.* Typhi carriage, the liver [23] and the bone marrow [24] have been demonstrated as alternative sites for persistence in human patients. In addition, macrophages in MLNs were identified as a reservoir in chimpanzees infected with *S.* Typhi [25].

The gall bladder may not serve as the primary persistence niche for NTS serovars in humans [9]. However, in *S.* Typhimurium infected mice, *Salmonella* can colonize the gall bladder and periodic reseeding of salmonellae from the gall bladder into the small intestine can occur via the bile, which might be followed by the excretion of the bacteria into the feces [4]. In long-term infections in mice, *Salmonella* are associated with DCs and macrophages in the lamina propria and MLNs [16,26]. Furthermore, *S.* Typhimurium has been shown to reside within hemophagocytic macrophages during persistent infection. These phagocytes are characterized by the ingestion of intact leukocytes and erythrocytes and are associated with an anti-inflammatory M2-like state [27–29]. A detailed analysis of the spleen of *S.* Typhimurium-infected mice showed that a small population of *Salmonella* located in the white pulp cannot be eradicated by antibiotic treatment and is responsible for persistent infection [30]. In addition, infection with *S.* Typhimurium entering a dormant or persistent state has been demonstrated in other cell types, such as cultured and *in vivo* fibroblasts [31–36], and in intestinal epithelial cell lines [37]. However, as the intestinal epithelium is renewed every 3–5 days, persistence mechanisms in the epithelium of the intestinal wall might differ from other cells/sites. Persistent infection in the intestine relies on continuous re-invasion of epithelial cells [16,38].

Numerous *Salmonella* serovars have the ability to form biofilms. Besides their importance for persistence in the environment [39], biofilms can also play important roles during infections. *S.* Typhi and *S.* Paratyphi are well-known to form biofilms on gallstones in the gall bladder which constitutes an increased risk of developing gall bladder carcinoma in humans [40–42]. In biofilms, bacteria produce extracellular polymeric substances that enhance bacterial survival by protecting against hostile environments, the host immune response, and antibiotics [43,44]. Biofilm growth exposes the bacteria to stressful conditions, such as starvation and oxygen deprivation, which may induce the SOS response and increase the resistance to various antibiotics [45]. Biofilms on implantable medical devices are a serious source for infections and difficult to impossible to eliminate. However, usually hospital-acquired bacteria such as *Pseudomonas aeruginosa* or *Staphylococcus epidermidis* are responsible for biofilm infections on implants whereas *Salmonella* biofilms are not commonly found on implants [44]. For a recent in-depth discussion of *Salmonella* biofilms in the context of persistent infections, we would like to refer the reader to the publication of Harrell and colleagues [44].

Besides biofilms, *Salmonella* can adopt multiple different growth phenotypes. For instance, small colony variants have been observed as a persister subpopulation in *in vitro* cultures including biofilms after antibiotic exposure [45]. They are characterized by slow growth leading to small-sized colonies on agar plates but can revert to wild-type-like colonies [45]. Furthermore, filamentous growth has been observed in cultures *in vitro* and in different cell types such as macrophages, fibroblasts, and epithelial cells [45–47]. Bacterial filamentation is a consequence of intracellular stress, such as oxidative and nitrosative stress, antimicrobial peptides, or antibiotic treatment, and is based on a transient cell division defect resulting in the incomplete septation of the replicating bacteria [48,49]. Following the re-establishment of favorable growth conditions, the bacteria resume septation into viable rod-shaped cells competent for infection [50,51]. Figure 1B illustrates the major different growth phenotypes including the persister phenotypes which has been extensively studied in macrophages as described below.

Macrophages as an intracellular niche

Macrophages of the MLNs are a primary site of *S.* Typhimurium colonization besides spleen, liver and gall bladder [26]. In long-term infections, the immunomodulatory environment of the liver favors *Salmonella* persistence [52]. Persistent *Salmonella* are found in M2-like macrophages in granulomas in spleen, liver, and MLNs. This macrophage subpopulation exhibits diminished antimicrobial functions, including reduced expression of *Tnfa* and *Nos2* as well as increased expression of *Il4ra,* in comparison to M1-type granuloma macrophages [26,53,54]. Tumor necrosis factor (TNF)-α restricts *Salmonella* persistence by suppressing M2 polarization and granuloma formation [53,55]. A recent publication showed that different macrophage subsets are infected during *Salmonella* infection and that a CD9-positive nonclassical monocyte-derived macrophage subset constitutes a replication niche for *Salmonella* [56].

Besides active replication in macrophages, *Salmonella* can enter a dormant, metabolically inactive state or a nongrowing, metabolically active state called persisters. Metabolically active persisters translocate *Salmonella* pathogenicity island (SPI)-2 effectors and can resume

Table 1

Main differences in the immune responses during acute and persistent *Salmonella* infections.

Host response	Acute infection	Persistent infection	References
Predominant type of immune response	Th1	Th2	[2,73,78]
TNF-α, IL-12, IFN-γ	+++	o/+	[26,53,73,74,76]
IL-4, IL-5, IL-10, IL-13	o/+	+++	[2,77]
Reactive oxygen/nitrogen intermediates	+++	o	[74–76]
Predominant macrophage polarisation	M1	M2	[53,57,83]
Killing of intracellular bacteria	+++	o/+	[78]

+++: high production, o/+: no/little production.

growth in a more favorable environment while dormant, metabolically inactive bacteria cannot resume growth and are possibly killed [57–59]. SPI-2 effectors reprogram macrophages towards an anti-inflammatory M2-like state and dampen the inflammatory immune response thereby enabling long-term survival. In the persistent state, the bacteria are exposed to a less stressful intracellular environment facilitating survival [60]. Toxin-antitoxin modules play an important part in persister formation [59,61,62]. In contrast to antibiotic resistance conferred by heritable genetic mutations, cells regrown from these persister subpopulations mainly remain antibiotic-sensitive [59]. Using *S.* Typhimurium and clinical isolates of invasive *S.* Typhimurium ST313, Helaine and colleagues recently demonstrated that antibiotic persistence and tolerance have different underlying mechanisms and that only the persister population is responsible for the relapse of infection [58]. Antibiotic persistence and tolerance have been recently comprehensively reviewed [63,64]. Slow growth seems to be a common principle of antibiotic persisters [65]. Different growth rates of *Salmonella* in the mouse spleen affect the sensitivity to antimicrobial chemotherapy generating partial tolerance and delayed eradication of moderately growing *Salmonella* [66]. Similarly, slow-growing *Salmonella* in DCs in the cecum lymph nodes were found to be phenotypically tolerant to antibiotic treatment [67]. Long-term infection also enhances the chances of horizontal gene transfer of plasmids encoding resistance genes [16] or the accumulation of mutations [9,58,68]. Thus, persisters do not only cause failure of antibiotic treatment but also facilitate the evolution of virulence [2,8] and antibiotic resistance as well as the spread of resistance plasmids [69,70]. The emergence of multidrug-resistant NTS and TS strains in recent years is a major concern and will further complicate and narrow potential therapeutic treatment options [71,72].

Immune response and *Salmonella* genes facilitating long-term infections

Salmonella infection initially elicits a strong Th1 immune response characterized by the secretion of TNF-α, interleukin (IL)-12, and interferon (IFN)-γ [73]. These cytokines have been shown to be crucial for host resistance to *Salmonella*, for instance, by stimulating the production of nitric oxide by iNOS [74–77]. The early pro-inflammatory Th1 response mediates the killing of intracellular bacteria, thereby reducing the bacterial burden [2,78] and inflammatory monocytes play a key role in controlling persistent infections [79]. In contrast, persistent *Salmonella* infections feature a downregulated Th1 response but an enhanced Th2 response, which is thought to be anti-inflammatory [78]. The Th2 response is characterized by IL-10, IL-4, IL-5, and IL-13. IL-10 dampens the production of reactive oxygen and nitrogen species as well as the release of TNF-α and IL-12 by macrophages. Moreover, increasing IL-10 levels result in declining IFN-γ levels, thus hampering macrophage-mediated killing of the bacteria and promoting persistence [77]. However, a basal level of IFN-γ is necessary to keep the persistent infection under control. For instance, administration of an IFN-γ neutralizing antibody to chronically infected mice results in massive bacterial replication and reactivation of systemic *S.* Typhimurium infection [26]. Similarly, TNF-α neutralization in persistently infected mice increases the bacterial load in systemic organs [53]. The immunosuppressive activity of regulatory T cells can tip the balance from clearing the infection to persistence [80]. Table 1 summarizes the characteristic immune responses observed during acute and persistent *Salmonella* infections.

Salmonella virulence relies to a large part on two type III secretion systems (T3SS-1 and T3SS-2) encoded on *Salmonella* pathogenicity island (SPI-1) and SPI-2. SPI-1 mediates the initial interaction of *Salmonella* with intestinal epithelial cells; the translocated effectors enable the pathogen to invade nonphagocytic cells and thus cross the intestinal epithelial barrier. In addition, GWAS studies revealed a role of T3SS-1 for *Salmonella* persistence *in vivo* [81]. Among the twelve SPI-1 associated genes shown to be required for persistent infection were genes encoding transcriptional regulators (e.g. HilA), structural components of the T3SS-1 secretion apparatus (e.g. SpaO), as well as T3SS-1 secreted effectors SopD and SteA. Moreover, the screen revealed a role of T3SS-1 translocated effectors and translocators SipB, SipC, and SipD for maintaining long-term infections [81] and the contribution of several SPI-1 and SPI-2 genes to persistence in the spleen after intraperitoneal infection of

mice was confirmed using single deletion mutants of *S.* Typhimurium [82].

Salmonella uses its SPI-2 effectors to reprogram macrophages from an 'inflammatory' M1 to an 'anti-inflammatory' M2-like state to avoid killing. M1 suppression and M2 polarization are independent of each other. M2 polarization is mainly driven by the effector SteE which antagonizes the TNF-α mediated restriction of M2 polarization [53,57]. SteE binds the host serine/threonine kinase GSK3 resulting in the activation of transcription factor STAT3 which initiates anti-inflammatory M2-type gene expression [83,84]. Of note, *S.* Typhi does not possess a *steE* gene [53] suggesting different, so far undefined mechanisms to manipulate macrophage polarization.

Besides SPI-1 and SPI-2, numerous *Salmonella* genes are involved long-term survival and prolonged infection reflecting the requirement to adapt to different environmental conditions intra- and extracellularly. Genes involved in attachment, resistance to antimicrobial peptides, resistance to bile, iron import, intracellular survival, and others are discussed in the recent reviews by O. Gal-Mor, and Ruby and colleagues [2,78]. For instance, several fimbriae binding to glycosylated structures contribute to long-term colonization of *S.* Typhimurium in the intestine [81,85,86]; reviewed in [87]. Another example is the PhoP/PhoQ system which regulates bacterial gene expression promoting intracellular survival and replication in macrophages, including resistance to cationic antimicrobial peptides. The PhoP/PhoQ system controls a variety of other virulence mechanisms dependent on the infected cell type [88].

Conclusions

Salmonella adapts to its extracellular or intracellular habitats with heterogeneous, transient and genetically determined phenotypes in order to persist in the host. Most of our mechanistic knowledge stems from experiments in mice or tissue culture models infected with *S.* Typhimurium. Although genetically closely related, infection experiments show that there is a wide spectrum how different *Salmonella* serovars interact with the mammalian host. As persistent infections are extremely difficult to treat, it will be important to determine the individual persistence mechanisms of specific serovars in order to identify novel therapeutic targets.

Conflict of interest statement

None.

Data Availability

No data were used for the research described in the article.

Acknowledgements

This work was funded by the Deutsche Forschungsgemeinschaft DFG Collaborative Research Center SFB900 "Chronic Infections: Microbial Persistence and its Control" TP8 (Projektnummer 158989968).

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