Molecular Mechanisms Used by *Salmonella* to Evade the Immune System

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Joaquín Bernal-Bayard[1]* and Francisco Ramos-Morales[2]

[1]Département de Microbiologie, Institut Pasteur, Paris, France.
[2]Departamento de Genética, Universidad de Sevilla, Seville, Spain.

*Correspondence: jbernal@pasteur.fr

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Abstract

Human and animal pathogens are able to circumvent, at least temporarily, the sophisticated immune defences of their hosts. Several serovars of the Gram-negative bacterium *Salmonella enterica* have been used as models for the study of pathogen–host interactions. In this review we discuss the strategies used by *Salmonella* to evade or manipulate three levels of host immune defences: physical barriers, innate immunity and adaptive immunity. During its passage through the digestive system, *Salmonella* has to face the acidic pH of the stomach, bile and antimicrobial peptides in the intestine, as well as the competition with resident microbiota. After host cell invasion, *Salmonella* manipulates inflammatory pathways and the autophagy process. Finally, *Salmonella* evades the adaptive immune system by interacting with dendritic cells, and T and B lymphocytes. Mechanisms allowing the establishment of persistent infections are also discussed.

Introduction

Salmonella spp. are pathogenic Gram-negative, rod-shaped, motile bacteria belonging to the family Enterobacteriaceae. The genus *Salmonella* includes the species *S. bongori* and *S. enterica*. *S. enterica* is further divided into several subspecies and more than 2500 serovars (Tindall *et al*., 2005). *Salmonella* can cause gastroenteritis, typhoid fever, abortion, and bacteraemia, depending on the serovar and the host. For instance, *S. enterica* subspecies *enterica* serovar Typhi (*S.* Typhi) is responsible for typhoid fever in humans, whereas *S. enterica* subspecies *enterica* serovar Typhimurium (*S.* Typhimurium) causes gastrointestinal inflammation in humans and a systemic typhoid-like disease in mice (Garai *et al*., 2012). *Salmonella* is a facultative intracellular pathogen that usually resides in a modified phagolysosome known as *Salmonella*-containing vacuole (SCV) during host infection. However, recent data indicate that this facultative intracellular pathogen has a distinct bimodal lifestyle in epithelial

cells, where, there are subpopulations of vacuolar and cytosolic *Salmonella* (Knodler *et al.*, 2010). Target cells include M cells, gut epithelial cells, dendritic cells (DCs), macrophages, monocytes, neutrophils, B cells, and T cells. During its passage through the host, *Salmonella* must resist or evade multiple levels of immune defence. Many virulence genes contribute to the survival of these bacteria inside the host. Some of them are clustered in horizontally acquired genomic regions known as *Salmonella* pathogenicity islands (SPIs) (Gerlach and Hensel, 2007). The largest and best known are SPI1, which is present in all the members of the genus, and SPI2, which is present in *S. enterica* but not in *S. bongori*. These SPIs encode two distinct type III secretion systems, type III secretion system 1 (T3SS1) and type III secretion system 2 (T3SS2), respectively (Galán and Curtiss, 1989; Ochman *et al.*, 1996; Shea *et al.*, 1996). These systems are able to inject directly into the eukaryotic host cells a number of bacterial proteins known as effectors (Ramos-Morales, 2012a). Collectively, these virulence factors contribute to invade host cells, interfere with host cellular functions, subvert immunity, establish an intracellular niche, and promote pathogen proliferation.

In this review we summarize the mechanisms of immunity that pathogens in general have to face and the specific strategies that *S. enterica* uses to deal with the different levels of defence of the host.

Overview of mechanisms of immune defence

Humans, like any other animal are confronted every day to a myriad of microorganisms. However, these potentially infectious agents do not cause disease regularly due to a series of defence mechanisms that have been shaped over millions of years of evolution.

Three levels of immune defence can be recognized (Delves *et al.*, 2011; Kenneth, 2011): (i) physical barriers, (ii) the innate immune system and (iii) the adaptive immune system.

Physical barriers

The best way to avoid infections is by keeping potentially infectious agents out of the body. Physical barriers are very effective for this purpose. They are represented by the skin and other epithelia covering the gastrointestinal, respiratory and urogenital tracts. These surfaces provide, not only mechanical, but also chemical and microbiological barriers.

The primary mechanical protection common to all epithelia consists of epithelial cells linked by tight junctions. The internal epithelia, or mucosal epithelia, are covered with mucous secretions. Mucus blocks the adherence of bacteria to epithelial cells. Microorganisms trapped within it can be removed by the movement of cilia, coughing or sneezing. In the intestinal mucosa, mucus is secreted by specialized goblet cells and creates a physical barrier against microbial pathogens (Liévin-Le Moal and Servin, 2006). Other general mechanical factors that contribute to this first level of defence are the flow of air or other fluids, like the tears in the eyes.

In addition to these physical obstacles, a chemical barrier to infections is provided by substances that kill microbes or that inhibit their growth. These chemical substances include a group of antibacterial enzymes present in body fluids, like lactoperoxidase in milk, lysozyme and secretory phospholipase A2 in tears, nasal secretions and saliva. Lysozyme breaks the peptidoglycan (PG) in the bacterial cell wall of Gram-positive and Gram-negative bacteria. Phospholipase A2 kills bacteria by hydrolysing phospholipids in the cell membrane. Antimicrobial peptides, like defensins, cathelicidins and histatins, represent another important

group of antimicrobial agents associated to epithelia. All of them are amphipathic peptides activated by proteolysis. Defensins and cathelicidins disrupt membranes and are toxic to many microorganisms, whereas histatins act against pathogenic fungi. Additional chemical barriers that are found by potential food pathogens include the acidity of the stomach pH and the bile in the intestine.

Microbiota is the microbial community composed of bacteria, protozoa, archaea, viruses and fungi that reside in different body niches. This microbiota competes with pathogenic microorganisms for nutrients and attachment sites. Therefore, the normal microbiota that is present in the intestine provides a microbiological physical defence against potential microbial pathogens that, like *Salmonella*, usually invade through the gut. In addition, some microbiota-produced metabolites affect the growth and virulence of pathogens. These metabolites include short-chain fatty acids, succinate, mucin O-glycans, molecular hydrogen, secondary bile acids, and the quorum sensing autoinducer AI-2 (Vogt *et al*., 2015).

Innate immune defence

The second level of defence is the innate immune response. This response occurs immediately after entry of a foreign agent into the body, but its effectiveness does not improve upon secondary encounter with the same agent. Microorganisms are recognized and killed by two main classes of phagocytic cells: polymorphonuclear neutrophils and macrophages. These cells carry pattern recognition receptors (PRRs) on their cytoplasmic membranes that adhere to pathogen-associated molecular patterns (PAMPs) on the microbe surface. PRRs include Toll-like receptors (TLRs), C-type lectin receptors (CTLRs), NOD-like receptors (NLRs), RIG-like receptors (RLRs) and scavenger receptors. PAMPs that activate TLRs include lipopolysaccharide (LPS), PG, lipoproteins, flagellin and other pathogen-derived ligands. Binding of a TLR to its ligand leads to activation of nuclear factor κB (NF-κB) and several members of the interferon-regulated factor (IRF) family of transcription factors. CTLRs are very diverse transmembrane proteins. RLRs are found in the cytoplasm and activate NF-κB and IRF3/4 to induce antiviral type I interferons in response to double-stranded RNA. Scavenger receptors recognize modified low-density proteins, LPS and other ligands, and some of them can cooperate with TLRs (Murshid *et al*., 2016). NLRs are soluble cytosolic proteins that usually are composed of three domains: an N-terminal domain that recruits proteases or kinases, a central oligomerization domain and C-terminal leucine-rich repeats (LRRs) that recognize PAMPs. NLRs exist in an autoinhibited conformation that is released upon binding to a PAMP, allowing oligomerization and recruitment of different host ligands depending on the particular NLR (Kim *et al*., 2016). The NLRs NOD1 and NOD2 induce autophagy to remove pathogens by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry. Autophagy is a conserved intracellular degradation pathway that involves the formation of double-membrane vesicles, known as autophagosomes, which deliver cytosolic components to the lysosome for degradation. Microbe-induced autophagy, or xenophagy, is important to restrict the growth of a number of intracellular pathogens, including *S.* Typhimurium (Gomes and Dikic, 2014). NOD1 and NOD2 activate NF-κB and mitogen-activated protein kinase (MAPK) signalling pathways, whereas NLRP2 and NLRP4 are negative regulators of the NF-κB pathway. Some NLRs, as well as absent in melanoma 2-like receptors (ALRs) and pyrin, act as inflammasome sensors (Sharma and Kanneganti, 2016). The inflammasome is mainly activated in myeloid cells as an essential component of innate immunity that promotes caspase-1-induced conversion of

procytokines into active IL-1β and IL-18. It can result in an inflammatory form of cell death known as pyroptosis. Inflammasomes are cytosolic protein complexes that usually contain a specific sensor, an adaptor molecule (ASC) and pro-caspase-1. Canonical inflammasomes contain NLRP1, NLRP3, NLRC4, AIM2 or pyrin as sensors. NLRP3 is also involved in a non-canonical inflammasome that activates pro-caspase-11 in response to Gram-negative bacteria. This inflammasome is primed by recognition of LPS through a TLR and type I interferon signalling (Rathinam and Fitzgerald, 2016).

The general consequences of binding to PRRs include the activation of macrophages and neutrophils to increase their phagocytic activity, and the release of cytokines and chemokines that amplify the immune response. Once inside the phagocytic cell, the microorganism can be killed by reactive oxygen or nitrogen intermediates, by preformed antimicrobials like the defensins mentioned above or a neutral protease. This takes place with the assistance of other factors like low pH, lysozyme and lactoferrin. Other important cells in the innate immune system are natural killer cells, which identify host cells expressing abnormal patterns of proteins and induce apoptosis of these cells.

In addition to the cellular components of innate immunity, the complement system and other soluble circulating defensive proteins integrate the humoral innate immunity. The complement system is a multicomponent enzyme cascade that facilitates phagocytosis and lysis of microorganisms. This system can be activated by the lectin, classical or alternative pathways. All of the aforementioned generate a C3 convertase that leaves C3b bound to the microbial surface and releases C3a. C3b acts as an opsonin that increases the ability of phagocytes to ingest bacteria. C3a, together with C5a, recruit phagocytic cells to the site of infection and promote inflammation. Complement activation also leads to the formation of a membrane-attack complex that causes cell lysis. In addition, complement activation together with products of activated mast cells promotes inflammation, a response that leads to local swelling, redness, pain and temperature elevation.

Adaptive immune response

Unlike innate responses, acquired or adaptive immunity is able to recognize specific antigens and develop a response against these pathogens. In contrast to innate immunity, adaptive immunity is different in each individual of a species and responds to specific antigenic challenges, displaying then a flexible spectrum of action, as it is able to recognize millions of different antigenic molecules. The two major varieties of lymphocytes, B-lymphocytes and T-lymphocytes, are essential to generate humoral and cellular immunity, respectively. B-lymphocytes are able to make antibodies, specific antigen-recognition molecules, known as immunoglobulins, which deal with extracellular infections. T-lymphocytes are involved in the control of intracellular infections. Their receptors recognize processed antigens in association with molecules of the major histocompatibility complex (MHC). T cells are classified in two categories: CD4 (helpers) and CD8 (cytotoxic) T cells, which are activated upon recognition of an antigen presented by major histocompatibility complex class II (MHC-II) and major histocompatibility complex class I (MHC-I), respectively by the TCR. While MHC-I is present on most cell surfaces, MHC-II is expressed on antigen presenting cells (APC).

A connection between the innate and adaptive immune systems at the level of cellular immunity is provided by DCs, which are relevant APCs. Stimulation of DCs by PAMPs triggers their maturation. Mature DCs present antigens to T cells via MHC molecules and

also provide co-stimulatory signals via B7 family ligands, promoting the activation of naive T cells. The interconnection between innate and adaptive immunity is also important for humoral immunity since the classical pathway to activate the complement requires immunoglobulin M (IgM) or immunoglobulin G (IgG).

Dealing with physical barriers

Acidic pH

Bacteria belonging to the genus *Salmonella* usually invade their hosts through oral ingestion of contaminated food or water. Lysozyme found in saliva is not a significant barrier for these pathogens since, like many other Gram-negative bacteria, they are capable of resisting it thanks to their outer membrane shield around the PG layer (Van Kesteren *et al.*, 1942; Masschalck and Michiels, 2003). Therefore, the first real challenge that these bacteria have to face upon ingestion is the acidic pH of the stomach of the host (Ramos-Morales, 2012b) due to gastric secretions and hydrochloric acid. The pH value of the stomach is variable: a pH of 1.3 was observed in healthy humans in the fasted state and of 4.9 after meal ingestion (Russell *et al.*, 1993). Values of 3.9 and 4.0 were measured in rats and mice, respectively, in the fasted state (McConnell *et al.*, 2008). This low gastric pH can quickly kill enteric bacteria, but different bacterial species have developed mechanisms to survive during passage through the stomach. When compared with *E. coli*, that resists extreme acid stress and can survive at pH 2 for hours, *S. enterica* is much less acid resistant (Koutsoumanis and Sofos, 2004; Lin *et al.*, 1995). However, *S.* Typhimurium possesses at least three mechanisms of acid tolerance response (ATR) (Audia *et al.*, 2001): (i) the exponential phase ATR is induced at pH 4.5–5.8 and allows subsequent survival at pH 3; (ii) the RpoS dependent stationary phase ATR is pH independent and is part of a general stress response; (iii) the RpoS independent stationary phase ATR is induced by exposure to pH 4.5 and provides longer tolerance to pH 3 than the RpoS dependent system. These tolerance systems require acid shock proteins whose synthesis is regulated by RpoS, the iron regulatory protein Fur or the two-component systems PhoQ/PhoP or EnvZ/OmpR (Audia *et al.*, 2001; Foster, 1993). Small RNAs (sRNAs) have recently been identified as major regulators of stress response networks. For instance, the sRNA DsrA influences the ATR and virulence in *S.* Typhimurium (Ryan *et al.*, 2016).

Bacteria that are able to survive the passage through the stomach must face new challenges in the intestine before establishing an intracellular niche. This includes mucus, bile, antimicrobial peptides and the resident microbiota (Fig. 6.1).

Mucus

Epithelial cells in the human gut are covered with mucous secretions that protect the epithelium from the environment. The mucus barrier is composed of at least two glycan layers, which in turn incorporate multiple layers of glycoproteins (mucin) and complex oligosaccharides (glycocalyx) that protect cells from the local environment and infection. Microbes are able to use the host glycan as nutrients that regulate bacterial infection and virulence (Marcobal *et al.*, 2013). Intestinal intracellular pathogens have to penetrate the mucin layers and subsequently gain access to the cell membrane. In order to achieve this, *Salmonella* uses a set of glycosyl hydrolases (GHs) that are able to degrade glycans. Recently, Arabyan *et al.* (2016) investigated the specific enzymes used by *Salmonella* to degrade the glycan. They

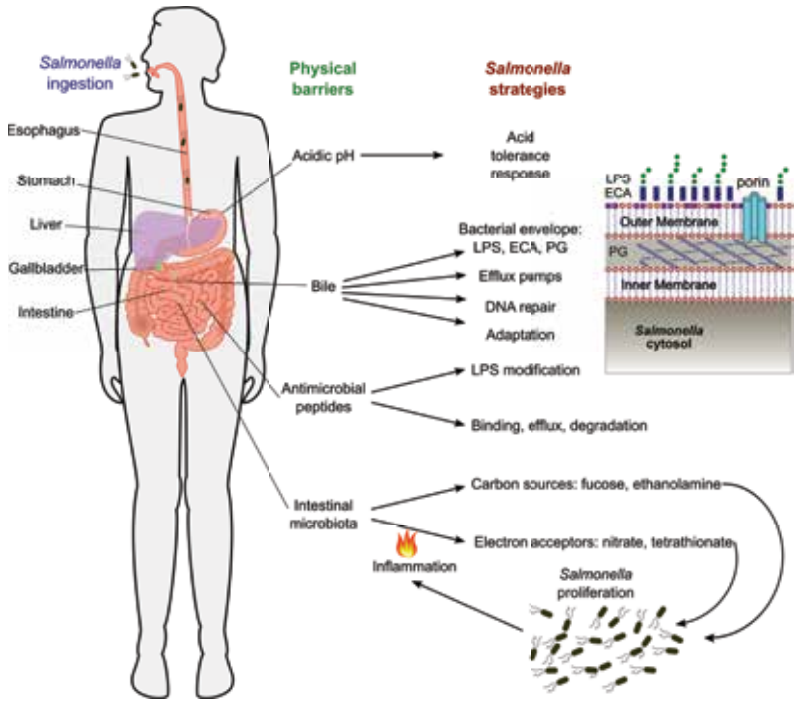

Figure 6.1 Schematic representation of *Salmonella* strategies to circumvent host physical barriers. After ingestion of contaminated food or water, *Salmonella* crosses the stomach where it is able to resist to acidic pH activating an acid tolerance response. Then *Salmonella* reaches the intestine where it has to face different chemical challenges, like the bile produced in the liver or antimicrobial peptides secreted by Paneth cells in the gut. In addition to that, *Salmonella* has to compete with the resident intestinal microbiota for essential nutrients. Interestingly, *Salmonella* triggers an inflammation response in the gut that offers different nutrients or adhesion receptor sites that can be exploited only by pathogens.

identified two specific glycan-degrading enzymes in *Salmonella*, NanH and MalS, as new virulence factors. These enzymes are expressed during *in vitro* infection of human colonic epithelial cells (Caco-2) and degrade the glycocalyx layer produced by these cells. The authors also analysed the host response to glycan degradation and they observed induced expression of host genes that hydrolyse mannose, fucose, and N-acetylneuraminic acid (Neu5Ac) from the glycan; as well as sialyltransferases, suggesting host glycan remodelling during infection (Arabyan *et al.*, 2016).

Bile

Bile is a secretory and excretory fluid produced in the liver whose composition varies depending on the individual's diet (Coleman, 1987). Secretory functions include: delivery to the intestinal tract of bile salts to aid fat digestion and absorption/secretion of polymeric IgA to prevent infection in the biliary and upper intestinal tracts. In addition, bile is the excretory vehicle of liver-derived metabolites of potentially toxic materials. Bile contains inorganic ions, bile salts, lipids (phosphatidylcholine, cholesterol) and proteins (plasma proteins, liver-specific proteins, polymeric IgA). It is a bactericidal agent that disrupts

bacterial cell membranes due to the detergent activity of bile acids (Begley *et al.*, 2005), causes DNA damage (Prieto *et al.*, 2004, 2006) and alters the conformation of proteins. *Salmonella*, however, resists bile through different mechanisms. First, the bacterial cell envelope acts as a barrier to membrane-active agents like bile salts. Two components of the outer membrane are important for bile resistance: the LPS (Crawford *et al.*, 2012; Gunn, 2000) and the enterobacterial common antigen (ECA) (Ramos-Morales *et al.*, 2003). Recently, it has been shown that exposure to the bile salt sodium deoxycholate is associated with changes in the structure of the second layer of the cell envelope, the PG, and that PG remodelling contributes to bile resistance (Hernández *et al.*, 2015). Once bile enters the cell, additional mechanisms are activated. Efflux pumps mediate the export of bile salts from the bacterial cytoplasm to the outside medium and are responsible for resistance to many different toxic compounds (Nishino *et al.*, 2006). AcrAB-TolC is the best characterized efflux system in enteric bacteria (Ma *et al.*, 1993, 1995; Nikaido *et al.*, 2008; Zgurskaya and Nikaido, 1999). AcrB is an inner-membrane protein, TolC is the outer membrane component and AcrA is located in the periplasm. The genes encoding these proteins are regulated by the transcriptional regulator RamA, which is synthesized in response to bile (Baucheron *et al.*, 2014). In addition, bile-induced DNA damages can be repaired by Dam-directed mismatch repair and by base excision repair, and the impairment of DNA replication rescued by SOS-dependent translation DNA replication and RecBCD-dependent recombinational repair (Prieto *et al.*, 2006). Finally, *Salmonella* is also able to adapt to grow in extremely high bile concentrations if previously exposed to sublethal doses. This is a transitory state that involves multiple changes in gene expression (Prouty *et al.*, 2004). The RpoS-dependent general stress response plays a crucial role in the adaptation of *Salmonella* to bile (Hernández *et al.*, 2012).

Antimicrobial peptides

Different families of antimicrobial peptides protect the intestine. Defensins and cathelicidins are of special interest in the context of gastrointestinal infections (Wehkamp *et al.*, 2007). They are produced in the intestinal epithelium either constitutively or in an inducible manner. For instance, Paneth cells of the small intestine express the most abundant, constitutively expressed defensins. Human cathelicidin is expressed by epithelial cells in the stomach, small bowel and colon. In this context, butyrate, a short-chain fatty acid produced in the colon by bacterial fermentation of dietary fibres, has been shown to induce expression of the human cathelicidin gene. *Salmonella* is able to sense sublethal concentrations of antimicrobial peptides and induce several mechanisms of resistance that involve modification of the LPS or sequestering, efflux and proteolytic degradation of antimicrobial peptides (Matamouros and Miller, 2015). Regulatory proteins involved in this process include the alternative sigma factor RpoE and the two-component or phosphorelay systems PhoQ/PhoP, PmrA/PmrB and Rcs. PhoQ is an inner-membrane histidine kinase that senses antimicrobial peptides (Bader *et al.*, 2003, 2005) and phosphorylates the response regulator PhoP. Activation of PhoP prevents dephosphorylation of PmrA (Kato and Groisman, 2004). This protein directly controls expression of genes involved in LPS modifications necessary for resistance to antimicrobial peptides. The Rcs system is a phosphotransfer cascade that responds to antimicrobial peptides through RcsF (Farris *et al.*, 2010). This system controls the synthesis of colanic acid capsule and the transcription of other genes necessary for resistance (Detweiler *et al.*, 2003). Modifications of the bacterial surface that contribute to antimicrobial peptide resistance include:

1 Regulation of O-antigen length by the PmrA/PmrB system (Farizano *et al.*, 2012; Pescaretti *et al.*, 2011).
2 Modification of the anionic phosphate groups in the core and lipid A regions of the LPS by addition of aminoarabinose and phosphoethanolamine. PmrA is involved in the regulation of these modifications as well as in the inhibition of the activity of LpxT, which is responsible for increasing the negative charge of LPS by adding an additional phosphate. Together these changes lead to a reduction of the negative charge of the outer membrane and of the electrostatic interactions with antimicrobial peptides (Gunn *et al.*, 1998; Herrera *et al.*, 2010; Jones *et al.*, 2008a; Lee *et al.*, 2004; Zhou *et al.*, 2001b).
3 Decrease in the fluidity of the outer membrane by the incorporation, catalysed by PagP, of an additional palmitate to lipid A and to phosphatidylglycerol (Bishop *et al.*, 2000; Dalebroux *et al.*, 2014; Guo *et al.*, 1998).

Additional resistance mechanisms involve binding, efflux or degradation of antimicrobial peptides. The two PhoP-regulated proteins Mig-14 and VirK are suggested to bind antimicrobial peptides to prevent their penetration into *Salmonella* (Brodsky *et al.*, 2005). The *sapABCDF* operon encodes an ABC transporter system involved in resistance to antimicrobial peptides (Parra-Lopez *et al.*, 1993). It has been proposed that the SapABCDF transporter system functions as a complex with SapG and SapJ to mediate both peptide and K^+ transport (Parra-Lopez *et al.*, 1994). Finally, PgtE is a post-transcriptionally regulated component of the PhoQ/PhoP regulon with protease activity against alpha-helical cationic antimicrobial peptides (Guina *et al.*, 2000).

Intestinal microbiota

Before invading appropriate host cells and becoming an intracellular pathogen, *Salmonella* has to compete with the resident intestinal microbiota. The lumen of the human gastrointestinal tract is rich in nutrients and provides an appropriate niche for a diverse community of bacteria (Bäckhed *et al.*, 2005). They increase in number from the stomach to the colon, where they reach concentrations around 10^{12} per gram of luminal content. Most colonic bacteria are members of the Firmicutes and Bacteroidetes phyla, whereas Proteobacteria, Actinobacteria, Fusobacteria and Verrucomicrobia are less abundant (Eckburg *et al.*, 2005). This microbiota helps to prevent infection by providing a physical barrier for the attachment of bacterial pathogens to the mucosal surfaces and competing with pathogens for essential nutrients (van der Waaij *et al.*, 1971). *Salmonella*, however, can exploit the host innate immune response and its own metabolic capacities to overcome, at least temporarily, this barrier (Khan, 2014). First, the initial growth of *Salmonella* in the gut is powered by Hyb hydrogenases that enable the bacteria to use hydrogen produced by the resident microbiota as a central intermediate of metabolism (Maier *et al.*, 2013). Using a mouse colitis model, it has been shown that inflammatory host responses triggered by *S.* Typhimurium virulence factors (T3SS effectors) shift the competition in favour of the pathogen (Stecher *et al.*, 2007). The mechanisms leading to this result are not completely understood and different explanations have been proposed: (i) inflammation involves release of antibacterial factors that may kill or delay the growth of specific members of the microbiota that would normally inhibit *Salmonella* growth; and (ii) the inflamed intestine could offer different

nutrients or adhesion receptor sites that can be exploited only by pathogens. In particular, reactive oxygen species generated by neutrophils during inflammation can react with endogenous thiosulfate to form tetrathionate. The *ttrRSBCA* locus, located in SPI2, confers to *S.* Typhimurium the ability to use tetrathionate as a terminal electron acceptor in anaerobic respiration (Winter *et al.*, 2010a). This confers a growth advantage because, unlike most bacteria in the gut, *S.* Typhimurium can use ethanolamine as a carbon source in the presence of tetrathionate as a respiratory electron acceptor (Thiennimitr *et al.*, 2011). It has also been suggested that the ability of *S.* Typhimurium to degrade fucose and anaerobically degrade 1,2-propanediol, probably using tetrathionate as a terminal electron acceptor, provides an advantage to this pathogen during competition with the microbiota (Staib and Fuchs, 2015). In addition, some *S.* Typhimurium isolates carry the gene *sopE* in a prophage. The product of this gene increases the severity of intestinal inflammation and drives the host to generate an additional terminal electron acceptor, nitrate, that suppresses genes responsible for utilization of energetically inferior electron acceptors such as tetrathionate (Lopez *et al.*, 2012). Stecher *et al.* (2012) described in an interesting study the discovery of a mechanism driving efficient conjugation between Enterobacteriaceae (*S.* Typhimurium and *E. coli*) in the host's intestine. Using a mouse colitis model, the authors observed parallel blooms of pathogenic *S.* Typhimurium and resident commensal *E. coli*. This phenomenon, probably due to dysbiosis of the anaerobic microbiota caused by *Salmonella* infection, led *E. coli* to account for > 80% of the total intestinal bacteria. In this scenario, the authors observed conjugative horizontal gene transfer efficiencies of 100%. This suggests that bacterial blooms occur after *Salmonella* infection fuels the reassortment of genetic material between different Enterobacteriaceae. This further implies that infected patients might enhance the spread of plasmid-encoded fitness, virulence- and antibiotic resistance determinants (Stecher *et al.*, 2012).

Evasion of the innate immune system

*Salmonella*e that are able to deal with the aforementioned physical barriers can invade the gut epithelium, preferentially in the distal ileum, using several routes. They usually invade microfold cells (M cells) that overlie Peyer's patches, but they can also induce their own uptake by epithelial cells or be engulfed by $CD18^+$ phagocytes, probably monocytes or dendritic cells (Watson and Holden, 2010). In a systemic infection, *Salmonella* can disseminate to internal organs [mesenteric lymph nodes (MLNs), liver and spleen]. Access to other cell types, like macrophages, could be a consequence of cell death induction (Fink and Cookson, 2007) and subsequent phagocytosis. Both intracellularly and during the transient extracellular passage from one host cell to another, *Salmonella* has to face and resist components of the innate immune system.

Salmonella finds cationic antimicrobial peptides not only in the intestinal lumen but also inside macrophages. Resistance mechanisms against this innate defence were described in the previous section. Interestingly, the PhoQ/PhoP two component system, that sense these antimicrobial peptides, are also additively activated by intracellular signals (acidic pH and divalent cation limitation). However the presence of antimicrobial peptides could be the most relevant signal for systemic virulence (Hicks *et al.*, 2015).

Manipulation of inflammatory pathways

Relevant PRRs for the recognition of *Salmonella* PAMPs are members of the TLR family, associated to host membranes, and members of the NLR family, that recognize the presence of PAMPs in the cytosol. This recognition leads to the activation of the NF-κB pathway (O'Dea and Hoffmann, 2010). Specific *Salmonella* virulence factors that enhance NF-κB activity are the T3SS1 effector SopE, which is recognized by NOD1 (Keestra *et al.*, 2013), and the T3SS2 effector SrfA, that promotes NF-κB activation by binding to TOLLIP, an inhibitor of IRAK1 (Lei *et al.*, 2016). This proinflammatory pathway can also be induced by the T3SS needle proteins PrgI and SsaG through TLR2 and TLR4 (Jessen *et al.*, 2014). In addition, SopE acts as a GTP exchange factor (GEF) for Rho family GTPases Cdc42 and Rac1, leading to activation of the c-Jun N-terminal kinase (JNK) pathway. Three different *Salmonella* proteins have been involved in inflammasome activation: (i) flagellin, an inducer of the NLRC4 inflammasome injected through T3SS1; (ii) PrgJ, a component of the basal body inner rod of T3SS1, also detected by NLRC4 (Miao *et al.*, 2010a); (iii) SopE contributes to inflammasome activation through its GEF activity, although the specific inflammasome sensor has not been determined (Hoffmann *et al.*, 2010; Müller *et al.*, 2009). NLRP3 has been suggested to have also an important role in innate immune defence against *S.* Typhimurium, since mice lacking both NLRP3 and NLRC4 are significantly more susceptible to infection (Broz *et al.*, 2010). It has been recently shown that NLRP3 associates with NLRC4 in macrophages infected with *S.* Typhimurium or transfected with flagellin, revealing an unexpected overlap between two distinct inflammasomes (Qu *et al.*, 2016). *S.* Typhimurium and other Gram-negative bacterial pathogens could induce a non-canonical inflammasome involving cytosolic recognition of LPS and activation of caspase-11 in mice and caspase-4 in humans (Broz and Monack, 2013a; Broz *et al.*, 2012; Casson *et al.*, 2015; Storek and Monack, 2015). This activation has been observed in the context of infections with mutants that aberrantly enter the cytosol (Aachoui *et al.*, 2013) after lysis of the SCV dependent on IFN-induced GTPases (Meunier *et al.*, 2014). However, wild-type *S.* Typhimurium can also lyse their nascent vacuole following invasion of tissue culture epithelial cells and enter the cytosol where it can eventually hyper-replicate (Birmingham *et al.*, 2006; Knodler *et al.*, 2014). Using a semi-quantitative single-cell analysis, Malik-Kale *et al.*,showed that although cytosolic hyper-replication occurs in less than 20% of infected epithelial cells, it accounts for the majority of net intracellular replication (Malik-Kale *et al.*, 2012). Interestingly, the transcriptional profiling of both intracellular bacterial subpopulations are different and it is known that cytosolic *Salmonella* are induced for T3SS1 and flagellated, whereas vacuolar bacteria are T3SS2-induced (Knodler *et al.*, 2010). Eventually, cytosolic hyper-replication leads to epithelial cell death via pyroptosis. This results in cell lysis, proinflammatory cytokine release and escape of the cytosolic bacteria into the extracellular space, providing a potential mechanism of dissemination (Knodler, 2015).

Anti-inflammatory response to *Salmonella* infection

Although an initial inflammatory response is beneficial for *Salmonella* to outcompete the microbiota, as the intracellular infection progresses, the pathogen still has mechanisms to inhibit inflammatory pathways (Fig. 6.2). Down-regulation of flagellin expression in systemic sites is a way to prevent NLRC4 inflammasome activation (Cummings *et al.*, 2006; Miao *et al.*, 2010b; Winter *et al.*, 2010b). Control of bacterial production of the tricarboxylic acid (TCA) cycle metabolite citrate may contribute to evade NLRP3 inflammasome

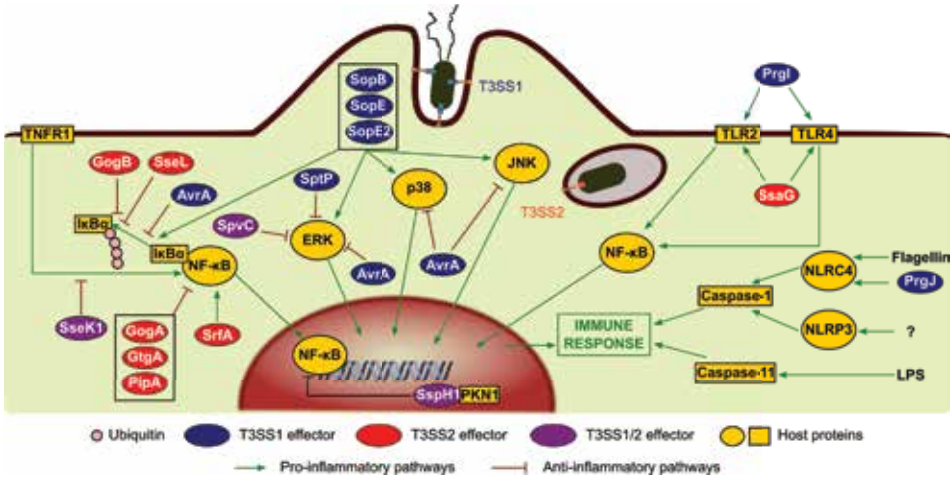

Figure 6.2 Schematic illustration of mechanisms of *Salmonella* to manipulate the host immune system by injecting effector proteins through two T3SS. *Salmonella* triggers its own internalization into the host cell in a T3SS1-dependent manner. Following internalization, *Salmonella* establishes its intracellular niche in a modified phagosome, called *Salmonella*-containing vacuole (SCV), where the pathogen manages to survive and proliferate. Biogenesis and maturation of the SCV depends on the activity of T3SS 1 and 2 effectors that manipulate the normal endocytic pathway to avoid the fusion and degradation by a lysosome. The actions of the T3SS proteins effectors on the inflammatory pathways to evade the host immune system are schematically represented.

activation in macrophages (Wynosky-Dolfi *et al.*, 2014). In addition, many T3SS effectors have an anti-inflammatory role. Both the T3SS1 effector AvrA and the T3SS2 effector SseL deubiquitinate IκBα and inhibit its degradation (Le Negrate *et al.*, 2008; Ye *et al.*, 2007). Binding of IκBα to NF-κB prevents its translocation to the nucleus. AvrA also inhibits JNK-induced signalling (Du and Galán, 2009; Jones *et al.*, 2008b; Wu *et al.*, 2012). GogA, GtgA and PipA are members of the same family of proteases that redundantly cleave specific members of the NF-κB family, including RelA (p65) and RelB (Sun *et al.*, 2016). SspH1 localizes to the mammalian nucleus and inhibits NF-κB-dependent gene expression through interaction with protein kinase 1 (PKN1) (Haraga *et al.*, 2006). The effector SptP consists of two domains. The N-terminal domain acts as a GAP for Cdc42 and Rac1 that mediates reversion of the effects of SopE on the actin cytoskeleton (Fu and Galán, 1999). The C-terminal domain possesses tyrosine phosphatase activity, which is (Kaniga *et al.*, 1996) involved in reversing the initial activation of the MAPK ERK, triggered by *Salmonella* infection (Murli *et al.*, 2001). SptP inhibits this MAPK pathway through inhibition of Raf1 activation in a process that involves both SptP activities (Lin *et al.*, 2003). This down-modulation of ERK activation could explain the contribution of SptP, together with SspH1, towards the inhibition of IL-8 production after invasion of intestinal epithelial cells (Haraga and Miller, 2003). Another effector involved in reduction of the production of inflammatory cytokines through the inactivation of ERK is SpvC. This effector is encoded in a virulence plasmid present in some *S. enterica* serovars and can be secreted by T3SS1 and T3SS2. SpvC inactivates ERK by irreversibly removing phosphate from a threonine residue in the conserved activation loop motif TXY using its phophothreoninelyase activity

(Mazurkiewicz *et al.*, 2008). Interestingly, during the interaction of *Salmonella* with the plant model *Arabidopsis*, SpvC interacts with and dephosphorylates activated MAPK6 to inhibit defence signalling (Neumann *et al.*, 2014). This suggests that some *Salmonella* effector proteins could have a conserved function during proliferation in different hosts. GogB is another anti-inflammatory effector that limits NF-κB activation by targeting the host SCF E3 ubiquitin ligase and inhibiting IκBα degradation (Pilar *et al.*, 2012). SseK1, an effector protein that can be secreted by T3SS1 and T3SS2 (Baisón-Olmo *et al.*, 2015), has a N-acetylglucosamine transferase activity that modifies the TNF-α receptor TNFR1 and its adaptor TRADD, preventing TNF-α mediated activation of NF-κB (Li *et al.*, 2013).

An important mediator of the anti-inflammatory effects of *Salmonella* is haem-oxygenase 1 (HO-1). HO-1 is an anti-stress regulatory molecule. It catabolyses the degradation of haem into biliverdin with the production of free iron and CO. Different studies have shown that HO-1 can be induced by a wide variety of bacteria. This is likely due to the increase in oxidative stress and inflammation (reviewed in Blancou *et al.*, 2011). Onyiah *et al.* (2013). using a colitis mouse model, showed that the enteric microbiota induces expression of HO-1 in mice and zebrafish. HO-1-derived CO enhances macrophage bactericidal activity and bacterial clearance *in vivo*. Similarly, others works have shown that an absence of HO-1 in humans and mice leads to chronic inflammation (Chung *et al.*, 2008; Yachie *et al.*, 1999), although the exact mechanisms remains unclear. One hypothesis emerged from the experiments performed by Choi and Lee, who showed a positive feedback of the system in which CO stimulates the synthesis of the anti-inflammatory cytokine interleukin 10 (IL-10) by macrophages (Otterbein *et al.*, 2000). IL-10 induces the expression of HO-1 potentially leading to a self-amplification of the anti-inflammatory effect (Lee and Chau, 2002). Others propose that HO-1 controls early innate immune response of *S.* Typhimurium-infected macrophages. In this study, a reduced survival of intracellular *Salmonella* in cells with low HO-1 expression was observed, probably due to the limited iron availability and to the activation of pro-inflammatory pathways that occurs in the absence of this enzyme (Mitterstiller *et al.*, 2016). On the other hand, *Salmonella*-infected macrophages with strongly up-regulated HO-1, provide a survival niche for *Salmonella* (Nix *et al.*, 2007). Thus, the induction of HO-1 expression has been suggested to be a strategy of the pathogen, given its harmful effects in the early control of *Salmonella* infection.

Another example of the immunosuppressive role of HO-1 was described by Riquelme *et al.* (2015), who suggest HO-1 prevents T-cell-mediated inflammatory disease by producing CO and impairing DC immunogenicity. CO has been shown to impair the mitochondrial function in DCs by reducing both the mitochondrial membrane potential and ATP production. This gas is able to impair the endo-lysosomal antigen trafficking route, reducing the ability of DCs to prime naive T cells. The role of HO-1 in immunity has been also studied in DCs, where CO is the molecule with the most prominent immunosuppressive capacity over their function (Rémy *et al.*, 2009; Tardif *et al.*, 2013). Contrary to this, other authors, like Zaki *et al.*, suggest that HO-1 contributes to improved control of *Salmonella* replication within macrophages by exerting a cytoprotective effect (Zaki *et al.*, 2009). Further studies are necessary to clarify the dual role of HO-1 in infections.

Autophagy

The relationship of *Salmonella* with autophagy is also of interest. During *S.* Typhimurium infection, T3SS1-mediated damage of the SCV allows ubiquitin and galectin-8 to target

bacteria inside the affected SCVs or free in the cytoplasm (Birmingham and Brumell, 2006; Birmingham *et al.*, 2006; Thurston *et al.*, 2012). These signals recruit essential anti-bacterial autophagy components including several cargo receptors, the kinase TBK1 and WIPI2, that restrict *Salmonella* proliferation (Radtke *et al.*, 2007; Thurston *et al.*, 2016, 2009, 2012). Autophagy has also a role in sealing damaged endosomal membranes, allowing T3SS2 induction inside the SCV (Kreibich *et al.*, 2015; Owen and Casanova, 2015). In epithelial cells, *Salmonella* induces the formation of ubiquitinated aggregates near the SCV in a T3SS2-dependent manner. These aggregates are recognized by the autophagy machinery. The T3SS2 effector SseL is a deubiquitinase whose activity lowers autophagic flux and favours intracellular *Salmonella* replication (Mesquita *et al.*, 2012). In a recent study that used a fibroblast-infected model, López-Montero *et al.* (2016) showed that *S.* Typhimurium develops membranous aggregates connected to the phagosome where the bacteria controls its progeny and establishes an intracellular persistent infection. In addition, T3SS2 mediates active suppression of autophagic signalling in macrophages through recruitment of focal adhesion kinase (FAK) to SCVs and activation of the Akt-mTORC1 signalling pathway (Owen *et al.*, 2014). The inhibition of autophagy by *Salmonella* also prevents the induction of a protective cytokine response mediated by interferon beta (IFN-β) (Owen *et al.*, 2016). Two recent studies show that SpvB, a T3SS2 effector with ADP-ribosyltransferase activity that prevents polymerization of G-actin into F-actin filaments (Hochmann *et al.*, 2006; Lesnick *et al.*, 2001; Margarit *et al.*, 2006; Tezcan-Merdol *et al.*, 2001), can inhibit autophagosome formation and increase inflammatory injury in zebrafish intestine (Li *et al.*, 2016) mammalian cells and mice (Chu *et al.*, 2016). This inhibition occurs at the early stage of autophagy via depolymerization of actin filaments.

The complement system

Finally, mechanisms to evade or interfere with the complement system have evolved in *Salmonella*. The human pathogen *S.* Typhi expresses the Vi capsular polysaccharide during transit through the intestine (Tran *et al.*, 2010). This polysaccharide has anti-inflammatory properties, (Sharma and Qadri, 2004) and also inhibits complement deposition (Looney and Steigbigel, 1986; Wilson *et al.*, 2011). This, thanks to a lack of hydroxyl groups available for ester formation with C3b (Heyns and Kiessling, 1967). Evolution of these immune evasive properties of *S.* Typhi led to inactivation of the *fepE* gene, encoding a regulator of very-long O-antigen chains (Crawford *et al.*, 2013). The surface protease PgtE of *S.* Typhimurium affects complement activity by cleaving C3b, C4b, C5, B and H complement components (Ramu *et al.*, 2007; Riva *et al.*, 2015). Although factors B and H have opposite effects on complement activation, the overall effect of PgtE favours protection of *Salmonella* against the host immune system, since less C3-derived fragments accumulated on *Salmonella* and the association with neutrophils was reduced (Riva *et al.*, 2015).

Mechanisms to escape adaptive immunity

In addition to mechanisms developed by *Salmonella* to escape from innate immunity, this intracellular pathogen has acquired different mechanisms to evade different adaptive immunity mechanisms.

Interactions of *Salmonella enterica* with dendritic cells

Innate and adaptive immunities are linked by DCs. Therefore, the activation of an effective adaptive immune response relies on efficient DCs capable of priming of naive T cells. This is one of the most relevant immune evasion strategies for *Salmonella*. This pathogen has developed molecular mechanisms to prevent the presentation of bacterial antigens that prime naive T cells and therefore avoid the initiation of the adaptive immunity.

In general, virulent strains of *Salmonella* are capable of modulating phagocytosis by DCs, avoiding lysosomal degradation and preventing antigen presentation to T cells. Here, we summarize the mechanisms developed by *Salmonella* to escape from acquired immunity. It is well known that *Salmonella* is able to induce phagocytosis on epithelial cells by active translocation of T3SS1 effectors which modulate the actin cytoskeleton. This results in internalization of *Salmonella* into the epithelial cell (Scherer *et al*., 2000; Steele-Mortimer *et al*., 2002; Zhou *et al*., 2001a). In contrast, the uptake of *Salmonella* by DCs is tightly regulated by effector proteins of T3SS1 (Bueno *et al*., 2010), being able to control the amount of bacteria that enter into the cells, avoiding then a massive immune response that would restrict *Salmonella* replication and dissemination. Additionally, Riquelme *et al.* showed that IgG-opsonized *Salmonella* are recognized by different receptors (FcyRIII receptors and others that have not been identified) expressed on DCs surfaces. These immune complexes are internalized and degraded by the lysosomal route, restoring its capacity to present *Salmonella* antigens to T cells and initiate an adaptive immunity response. However, the exact molecular mechanism with which IgG interferes with the secretion of *Salmonella* virulent effectors or impair its capacity to evade capture in DCs remains unclear (Riquelme *et al*., 2012).

After internalization, establishing a systemic infection depends on the pathogen's capacity to survive inside the host cells and to evade the immune response. In that sense, there are many studies on *Salmonella* virulence proteins that contribute to intracellular survival in DCs and dissemination in the host (Albaghdadi *et al*., 2009). One of the most relevant strategies developed by *Salmonella* to survive and replicate intracellularly is the disruption of the host endocytic trafficking machinery to avoid its lysosomal degradation within the SCV. The ability to survive in DCs is dependent on SPI2 effectors (Albaghdadi *et al*., 2009; Halici *et al*., 2008; Jantsch *et al*., 2003), which are injected to DCs cytosol from the SCV through a T3SS2. In fact, T3SS2 mutants show a reduced capacity to survive inside DCs and are attenuated in mice (Tobar *et al*., 2006). However, there are several examples of T3SS1 effectors that participate in avoiding lysosomal fusion. Among these SPI1 effectors, SopB and SopE were found to play a role in SCV maturation (Hernandez *et al*., 2004; Mukherjee *et al*., 2001). SopB is a phosphatase that mediates phosphatidylinositol 3-phosphate production in the SCV, through Rab5 recruitment to the SCV and its effector Vps34 (Mallo *et al*., 2008). Another example is SopE, which acts as a Rab5-specific exchange factor and mediates the recruitment of Rab5 in the GTP form in the SCV (Mukherjee *et al*., 2001). There are also examples of T3SS2 effectors that impair trafficking of endocytic cargo to lysosomes. The effector SopD2 directly binds and inhibits the host GTPase Rab7, a regulatory switch central to endocytic trafficking and phagosome–lysosome fusion. Consequently, this limits Rab7 interaction with its dynein- and kinesin-binding effectors RILP and FYCO1. This in turn disrupts the regulation of microtubule motors (D'Costa *et al*., 2015). SifA is another SPI2 effector required for bacterial survival inside DCs that alters lysosomal function

(Beuzón *et al.*, 2000; Boucrot *et al.*, 2003; Petrovska *et al.*, 2004). McGourty *et al.* showed that SifA prevents the delivery of hydrolytic enzymes to the SCV by inhibiting Rab9-dependent retrograde trafficking of mannose-6-phosphate receptors (MPRs). This requires binding of SifA to its host cell target SKIP. Translocated SifA forms a stable complex with SKIP and Rab9 in infected cells. Sequestration of Rab9 by SifA-SKIP accounts for the effect of SifA on MPR transport and lysosome function (McGourty *et al.*, 2012). There are other effectors of the T3SS2 that are involved in DC intracellular survival of *Salmonella* like SseJ, SseF, SspH2 and PipB2. Strains lacking these effectors show reduced survival inside DCs but the specific role of each of these virulence proteins remains unclear (Halici *et al.*, 2008).

One of the suggested strategies used by *Salmonella* to avoid antigen presentation is inhibition of lysosomal degradation (Tobar *et al.*, 2006, 2004). There are studies that suggest that the PhoQ/PhoP regulatory system is involved in this escape mechanism. This because *Salmonella* strains with mutations in the PhoQ/PhoP system are not able to escape from lysosomal degradation and therefore do not interfere with antigen processing and presentation (Niedergang *et al.*, 2000). Other studies report that *Salmonella* regulates the expression of MHC class II antigens by polyubiquitination of HLA-DR. (Lapaque *et al.*, 2009). These mechanisms rely on virulence factors encoded in SPI2. T3SS2 effectors like SifA, SlrP, SspH2, PipB2 and SopD have been shown to participate in this evasion process since they are required for the inhibition of MHC-II-dependent antigen presentation in DCs (Halici *et al.*, 2008). The ability of *Salmonella* to prevent antigen presentation by DCs seems to depend exclusively on SPI2-related proteins, since a mutant strain lacking the T3SS1 is still able to avoid antigen presentation by DCs (Bueno *et al.*, 2010). Importantly, this feature seems to be host specific and restricted to serovar Typhimurium, since other serovars like *S. enteritidis* and *S.* Typhi are not able to interfere with this function in murine DCs (Bueno *et al.*, 2008).

S. Typhimurium can interfere with the host's immune response during oral infection of mice through selective killing of $CD8\alpha^+$ DCs in a process that is dependent on MyD88 and TNFR1 (Sundquist and Wick, 2009). It has been recently shown that the pR_{ST98} plasmid of *S.* Typhi may also influence maturation, survival and cytokine production of DCs, preventing activation of T-cell-mediated immunity against antigens derived from this pathogen (Wei *et al.*, 2015).

Another strategy used by virulent *Salmonella* is based on down-regulation of flagellin, a target of the innate and adaptive immune responses during infection. This strategy consists in preventing T-cell activation by the active reduction of the availability of bacterial antigens for presentation to T cells (Alaniz *et al.*, 2006). Some data suggest that flagellin is differentially expressed by *Salmonella* populations infecting Peyer's patches (Cummings *et al.*, 2006). Furthermore, intracellular *Salmonella* can make flagellin unavailable for antigen processing by DCs (Alaniz *et al.*, 2006). Similar studies showed that during low-dose *Salmonella* infections, the microbe evades activation of flagellin-specific CD4 T cells (Srinivasan *et al.*, 2004).

Direct interactions of *Salmonella enterica* with T cells

Examples of *S.* Typhimurium directly blocking T-cell proliferation are prevalent in the literature. This inhibition was observed in both $CD4^+$ and $CD8^+$ T cells and was due to a down-regulation of TCR expression β-chain, which interferes with the first step in T-cell clonal expansion (van der Velden *et al.*, 2005, 2008). The authors of the aforementioned study also identified the enzyme L-asparaginase II as responsible for this inhibitory effect.

The activity of this enzyme causes depletion of exogenous L-asparagine, leading to down-regulation of TCR-β expression, suppression of T-cell blastogenesis, blockade of cytokine production, and, ultimately, inhibition of T-cell proliferation (Kullas *et al.*, 2012). In agreement with this, the cytolytic capacities of $CD8^+$ T cells have been shown to be initiated by TCR interactions (Lewinsohn *et al.*, 2011).

The phagosomal lifestyle of *S.* Typhimurium seems to be responsible for delayed MHC-I dependent antigen presentation and delayed expansion and contraction of the $CD8^+$ T-cell response. Interestingly, even memory $CD8^+$ T cells failed to undergo rapid expansion in response to infection with *S.* Typhimurium expressing the model antigen OVA (Luu *et al.*, 2006).

Additionally, *Salmonella* is able to induce apoptosis of Ag-specific CD4 T cells (Srinivasan *et al.*, 2009). This is driven by SPI-2 virulence genes. *In vivo* studies by Ertelt *et al.* suggested that *Salmonella* undergoes, in a SPI-2 dependent manner, a selective culling of activated $CD4^+$ T-cell subsets, which re-shapes the repertoire of antigen-specific T cells that persist later after infection (Ertelt *et al.*, 2011).

Non-cognate activation of T cells

In addition to conventional activation by cognate peptide–MHC complexes presented by DCs, T cells can be indirectly stimulated (bystander stimulation) by *Salmonella*. This alternative pathway involves the well-characterized TLRs and NLRs, as well as other less known PRRs. It results in production of inflammatory cytokines that prime T cells at the site of infection (Broz and Monack, 2013b). Indeed, *Salmonella* has been proposed as a particularly appropriate model pathogen for study of non-cognate CD4 T-cell responses based on (i) strength of the Th1 response during infection, (ii) the requirement for CD4 T cells in bacterial clearance and (iii) the well-characterized inflammatory response to conserved molecular patterns induced by *Salmonella* infection (O'Donnell and McSorley, 2014).

B lymphocytes' role

B lymphocytes have critical roles as positive and negative regulators of immunity. For instance, B cells have important contributions in acquired immunity against *Salmonella* infections, being essential for immunity during secondary challenge (McSorley and Jenkins, 2000). Several studies describe antibody dependent (Mastroeni *et al.*, 1993; McSorley and Jenkins, 2000; Mittrücker *et al.*, 2000) and antibody independent *Salmonella* specific B cells responses (Barr *et al.*, 2010; Mastroeni *et al.*, 2000; Ugrinovic *et al.*, 2003). These studies suggest that B cells can work as APCs and have a key role in the production of inflammatory cytokines during *Salmonella* infection. The immunosuppressive role of B lymphocytes, classically associated with B cell-derived IL-10, is related to the regulation of autoimmune disease. This IL-10 also increase susceptibility to pathogens (Fillatreau, 2011; Fillatreau *et al.*, 2008). In a recent study, Shen *et al.* described interleukin 35 (IL-35)-producing B cells as novel key players in the negative regulation of immunity (Shen *et al.*, 2014). The authors showed that mice whose B cells did not express IL-35, displayed improved resistance to *S.* Typhimurium infection. The increased immunity found in these mice was associated to higher activation of macrophages and T cells, as well as an increased stimulatory function of B cells as APCs. The data presented in this work demonstrate that B cells can inhibit antimicrobial immunity through production of IL-35 (Shen *et al.*, 2014).

One of the strategies developed by *Salmonella* to evade the action of B cells is to delay the formation of the germinal centre, which is necessary to produce high-affinity antibodies against a specific pathogen (Cunningham *et al.*, 2007). It has been shown that during *Salmonella* infection germinal centre formation and affinity maturation are delayed until the second month of infection. This differs greatly with conventional 7 day functional germinal centre formation. Furthermore, high-affinity antibodies start to be produced during the second week after immunization.

Persistence

One important aspect of typhoid *Salmonella* is its capacity to establish chronic infections. After adequate treatment the majority of patients who present acute typhoid fever recover; however, a significant percentage of patients (1–6%) develop a chronic infection (Levine *et al.*, 1982; Monack *et al.*, 2004a). These patients become asymptomatic chronic carriers and sporadically shed bacteria in their stool, allowing the pathogen to close its life cycle and to be transmitted to new hosts (Monack *et al.*, 2004b). Despite the importance of *Salmonella* chronic infections, relatively little is known about the host immune response or virulence mechanisms that characterize long-term systemic infections. Since the majority of chronically infected patients are asymptomatic, the identification of potential targets is challenging (Shpargel *et al.*, 1985; Sinnott and Teall, 1987). However, it is interesting to note that gut persistent infections are *Salmonella* pathogenicity island 1 (SPI-1) and *Salmonella* pathogenicity island 2 (SPI-2) dependent (Lam *et al.*, 2014). One study suggested that the human carrier state may be associated to an ineffective immune response (Thompson *et al.*, 2009).

In this section we describe some recent advances with respect to *S.* Typhi and *S.* Typhimurium persistent host colonization.

Biofilm formation

Biofilm development is an important component of bacterial survival and a common source of persistent infections (Costerton *et al.*, 1999). *Salmonella* are able to form biofilm in both biotic and abiotic surfaces. Moreover, there is a clear correlation between chronic infection and bacterial biofilm formation on the surface of gallstones (Gonzalez-Escobedo *et al.*, 2011; Prouty *et al.*, 2002). Epidemiological studies have revealed a strong association between chronic carriers of *S.* Typhi and gallstones (Schiøler *et al.*, 1983). Although, 90% of chronically affected individuals have gallstones (Karaki and Matsubara, 1984) or other biliary track pathologies, this is not required to develop a carrier state (Monack *et al.*, 2004b). *Salmonella* biofilms have been found on gallstones in both *S.* Typhi patients and a mouse model of chronic *S.* Typhimurium infection (Crawford *et al.*, 2010), suggesting both serovars share this biofilm strategy to survive in their host. Interestingly, bile has been shown to enhance *S.* Typhi biofilm formation (Gonzalez-Escobedo *et al.*, 2011). Consistently, *S.* Typhi exhibits specific binding to cholesterol-coated surfaces, like gallstones (Crawford *et al.*, 2008). FliC and OmpC are two proteins that were described to have a key role on initial adhesion to cholesterol-coated surfaces (Crawford *et al.*, 2010). A hallmark of biofilm formation is the self-production of an extracellular matrix that ensures the integrity of the biofilm. The biofilm matrix is composed of EPS (extracellular polymeric substances) and water. Some components of biofilm EPS like cellulose, colonic acid and O antigen capsule are crucial for *S.* Typhi persistence and biofilm development (Crawford *et al.*, 2008; Prouty

and Gunn, 2003; Prouty *et al.*, 2002). Some studies show that bile present in the gallbladder induces the production of O-antigen, which facilitates *S.* Typhi biofilm formation on human gallstones (Crawford *et al.*, 2008; Hall-Stoodley and Stoodley, 2009).

Persistence in immune cells

The study of the molecular mechanisms underlying persistent infections is limited by the lack of appropriate animal models that resist challenges using virulent strains of *Salmonella*. A suitable mice model was established in 2004 to study *S.* Typhimurium persistent infections (Monack *et al.*, 2004b). In this model virulent *S.* Typhimurium strains cause long-term chronic infections. The persistent bacteria can then be monitored within macrophages in the MSNs up to 1 year post inoculation (Monack *et al.*, 2004b). In addition to that, *in vitro* assays showed that hemophagocytic macrophages might represent a survival niche for *Salmonella* (Nix *et al.*, 2007). Another study showed that persistent bacteria are sequestered within macrophages in systemic tissues, where they do not replicate and seem to be in a dormant-like state. This supports the idea of macrophages as a reservoir for persistent bacteria (Helaine *et al.*, 2010). Another study published recently, showed that *S.* Typhimurium preferentially associates with anti-inflammatory/M2 macrophages at later stages of infection in mice (Eisele *et al.*, 2013). This subset of macrophages does not express many of the defence mechanisms needed to eliminate invading microbes. Moreover, there are pathogens that have developed strategies to induce polarization of cells towards the M2 phenotype as a virulence mechanism (Jensen *et al.*, 2011).

In addition to macrophages, it has been shown that *Salmonella* remains for a long period of time within plasma cells, bone marrow B cell precursors, and all B cell subsets from the spleen (Castro-Eguiluz *et al.*, 2009; López-Medina *et al.*, 2015a). The ability of *Salmonella* to persist within B cells suggests that it developed a $CD8^+$ T-cell response evasion mechanism. In fact, *Salmonella* infection results in the enhanced expression of PD-L1 in B cells (López-Medina *et al.*, 2015a). PD-L1 is a member of the programmed death-1 (PD-1)/programmed death-ligands (PD-Ls) pathway that can turn off or reduce TCR signalling by recruiting phosphatases like SHP-1 and SHP-2 (Chemnitz *et al.*, 2004; Freeman *et al.*, 2000; Sheppard *et al.*, 2004). The activation of the PD-1 : PD-Ls axis leads to impairment of CD8 T cells during *Salmonella* infection (López-Medina *et al.*, 2015b).

T3SS effectors and persistence

In order to shed light on the molecular mechanisms involved in persistence, Monack's group performed a genome-wide screen to identify *Salmonella* genes required for persistent infection of the mice (Lawley *et al.*, 2006). The screen revealed that SPI1 was necessary to maintain a persistent infection for at least 1 month post challenge. The authors confirmed that SPI1 effectors SipB, SipC, and SipD, which have a role on invasion or translocation of other effectors, contribute to establish a persistent infection. Similarly, another study showed that SPI1 is required for persistently infected mice to transmit *S.* Typhimurium to naive cage-mates (Lawley *et al.*, 2008). Data from the genome screen mentioned above also showed that the SPI2 effector SseI was required for maintaining a long-term systemic infection. A follow-up study showed that SseI modulates macrophages and DCs by directly binding to IQGAP1, an important regulator of host cell migration (McLaughlin *et al.*, 2009). So, SseI blocks somehow the migration of macrophages and DCs interfering with the host immune system to clear systemic bacteria.

Other elements that have been shown to contribute to persistent *Salmonella* infection are those that protect against host-derived antimicrobial peptides. Examples of these factors are PgtE, a modifier of the bacterial outer surface membrane (Lawley *et al*., 2006), and Mig-14, which provides resistance against anti-microbial peptides like VirK, RcsC, and YdeI (Erickson and Detweiler, 2006), both of them regulated by the PhoP/PhoQ system (Bader *et al*., 2005).

Persistence and metabolism

Recently, a pioneering study described how bacteria modulate the host cell metabolism to create an appropriate environment for long-term infection (Eisele *et al*., 2013). Firstly, the authors observed that PPARδ, which is a transcriptional factor involved in fatty acid metabolism, is up-regulated in *Salmonella* infected M2 macrophages. This situation produces a shift in the metabolic state of the cell that leads to an increase in the level of glucose available to bacteria. Moreover, the authors show that pharmacological activation of PPARδ increases glucose availability and enhances bacterial replication in macrophages, while *Salmonella* fails to persist in macrophage lines lacking PPARδ. All together, these data, support the idea that *Salmonella* has evolved to survive long periods of time in M2 macrophages by harnessing the unique metabolism of these cells (Eisele *et al*., 2013).

Another relevant topic concerns genetic adaptation of bacteria during a persistent infection. A recent study describes the acquisition of adaptive mutations that facilitate persistence and survival in the host over the course of an infection (Søndberg and Jelsbak, 2016). In this work, the authors used a model of mice infected chronically with *S.* Typhimurium. They observed that bacteria acquire distinct single nucleotide polymorphisms (SNPs) in known regulators of metabolic and virulence genes. One such difference, the *kdgR*-SNP was confirmed to confer selective advantage during chronic infections and constitutes a true patho-adaptive mutation. Thus, the results provide evidence for rapid genetic adaptation to the host of *S.* Typhimurium during persistent infection (Søndberg and Jelsbak, 2016).

A non-mutational way for *Salmonella* to cause persistent infections even after antibiotic exposure is through the production of persister cells. These are rare cells, produced by all bacterial populations, that transiently become multidrug tolerant. It is thought this could be due to a state of low metabolic activity that leads to dormant or slow-growing cells (Bigger, 1944; Helaine and Kugelberg, 2014; Maisonneuve and Gerdes, 2014). *Salmonella* persisters are part of a non-replicating population formed immediately after uptake by macrophages and are induced by vacuolar acidification and nutritional deprivation (Helaine *et al*., 2014). Furthermore, the majority of the 14 toxin–antitoxin (TA) modules contributed to intracellular persister formation. A more comprehensive analysis of *S.* Typhimurium TA modules identified multiple toxins with anti-proliferative activity A selected group of the corresponding TA modules were important for survival of intracellular bacteria inside fibroblasts (Lobato-Márquez *et al*., 2015). One of these toxins, TacT, is an acetyltransferase that blocks the primary amine group of amino acids on charged tRNA molecules, thereby inhibiting translation and promoting persister formation (Cheverton *et al*., 2016).

Transmission

During systemic salmonellosis, the life cycle completed when the pathogenic bacteria leave the former host to infect a naive one. This highlights the main issue with persistent infections: the constant shedding of virulent bacteria on faeces allows for propagation and maintenance

within a population. In a chronic infection scenario, *Salmonella* can persist in MLNs, bone marrow, and gallbladder, and periodically discharge *Salmonella* from the gallbladder to the small intestine in bile. The molecular mechanisms that control transmission of bacteria from host reservoirs are poorly understood. However, an interesting study proposed that host transmission of *S.* Typhimurium is controlled by virulence factors and indigenous intestinal microbiota. The authors described a model in which persistently infected mice provide a natural mode of *S.* Typhimurium transmission. In this context, a subgroup of mice (30%), called 'supershedders' was found to shed high levels of *S.* Typhimurium in their stools, leading to rapid transmission of the infection to naive hosts (Lawley *et al.*, 2008). The authors showed that the development of the supershedder phenotype depends on SPI1 and SPI2. Moreover, treatment with antibiotics induced the supershedder state in mice, suggesting that the indigenous intestinal microbiota has a role in controlling this phenotype.

Concluding remarks

We have described recent advances concerning the strategies used by *Salmonella* to evade both innate and adaptive immune responses. Most of these evasion mechanisms are directly related to T3SS1 and 2 and to the different effectors injected by them (Table 6.1). In the last two decades more than 40 effectors have been identified, and the biochemical activity and host targets for some of them have been successfully characterized. However, the precise role of many of them and their contribution to the relationship between *Salmonella* and its hosts at the cellular and systemic level remain unknown. A better understanding of the specific functions of these effector proteins will allow us to unravel the complex network of action of these molecules and to decipher the underlying mechanisms that enable *Salmonella* to evade the host immune system.

For instance, we have to define the role of T3SS effectors in establishing different intracellular lifestyles and decipher which signals enable *Salmonella* to survive intracellularly versus proliferate and escape, depending on the cellular type.

Another challenge is to improve our understanding of *Salmonella* control of the fusion of SCV to the lysosome. The identification of new and appropriate SCV markers and the development of new imaging technologies will improve the detection of intracellular *Salmonella* and ultimately help us to answer these questions.

One of the most significant challenges concerns host specificity achieved by different *Salmonella* serovars. There is an obvious shortage of appropriate models. For example, there is no suitable animal model for the typhoid human pathogen *S.* Typhi. Therefore, *in vivo* studies for this serovar are deficient. Researchers tend to use the *S.* Typhimurium-infected mouse model to study systemic typhoid-like disease. However, extrapolation of results obtained in *S.* Typhimurium to *S.* Typhi is not always possible. The development of new animal and cellular models are slowly helping us surmount this lack of appropriate tools. One example is the recent work of Fresnay *et al.*, who established a controlled human infection model with wt *S.* Typhi using cutting-edge multichromatic flow cytometry to analyse the pre-challenge immunological status and its correlation with the subsequent clinical outcome (Fresnay *et al.*, 2016). Specifically, the authors investigated the relationship between *S.* Typhi-specific $CD8^+$ T-cell responses before exposure to wt *S.* Typhi and clinical outcome, i.e. whether the participants who were challenged developed disease or not. They observed higher baseline levels of multi functional *S.* Typhi-specific $CD8^+$ T in patients who did not

Table 6.1 Role of T3SS effectors in *Salmonella* evasion of the immune system

Effector	T3SS	Interaction with the immune system	References
AvrA	1	Inhibits NF-κB and JNK pro-inflammatory pathways	Collier-Hyams *et al*. (2002), Jones *et al*. (2008b)
CigR	2	Involved in biofilm formation	Yin *et al*. (2016)
GogA		Inhibits inflammation by targeting NF-κB signalling	Sun *et al*. (2016)
GogB	2	Limits inflammation preventing NF-κB	Pilar *et al*. (2012)
GtgA	2	Inhibits inflammation by targeting NF-κB signalling	Sun *et al*. (2016)
GtgE	1/2	Counters a Rab32-dependent host defence pathway	Spanò *et al*. (2016)
PipA		Inhibits inflammation by targeting NF-κB signalling	Sun *et al*. (2016)
PipB	2	Role in iNOS production in chicken oviduct epithelial cells	Li *et al*. (2009)
PrgJ	?	Activates caspase-1 through NLRC4	Miao *et al*. (2010)
PipB2	1/2	Inhibits dendritic cell migration	McLaughlin *et al*. (2014)
SifA	2	Inhibits dendritic cell migration	McLaughlin *et al*. (2014)
SipA/ SspA	1	Elicits accumulation of PERP to the apical surface of colonic epithelial cells that leads to inflammatory responses	Hallstrom *et al*. (2015)
SipB	1	Contributes to activation and release of IL-18	Dreher *et al*. (2002)
SipC/ SspC	1	Elicits accumulation of PERP to the apical surface of colonic epithelial cells that leads to inflammatory responses	Hallstrom and McCormick (2016)
SipD	1	Triggers cell death in macrophages	Arizmendi *et al*. (2016)
SlrP	1/2	Inhibits dendritic cell migration	McLaughlin *et al*. (2014)
SopA	1	Stimulates inflammation targeting TRIM56 and TRIM65	Kamanova *et al*. (2016)
SopB	1	Inhibits production of mitochondrial superoxide ROS Activates MAPK and NF-κB signalling through stimulation of Rho-family GTPases	Bruno *et al*. (2009), Ruan *et al*. (2016)
SopD	1/2?	Contributes to PMN migration and fluid secretion in bovine intestine	Zhang *et al*. (2002)
SopD2	2	Counters a Rab32-dependent host defence pathway	Spanò *et al*. (2016)
SopE	1	Induces caspase-1 dependent pro-inflammatory responses. Activates MAPK and NF-κB signalling through stimulation of Rho-family GTPases	Bruno *et al*. (2009), Hoffmann *et al*. (2010), Müller *et al*. (2009)
SopE2	1	Activates MAPK and NF-κB signalling through stimulation of Rho-family GTPases	Bruno *et al*. (2009)
SpiC/ SsaB	2	Inhibits dendritic cell migration	McLaughlin *et al*. (2014)
SptP	1	Inhibits p38 MAPK signalling. Suppresses degranulation of local mast cells	Choi *et al*. (2013), Tenor *et al*. (2004)

Table 6.1 Continued

Effector	T3SS	Interaction with the immune system	References
SpvB	2	Contributes to gut inflammation through a T3SS2-dependent pathway	Käppeli *et al.* (2011)
SpvC	1/2	Anti-inflammatory in mice. Interferes with immunity in plants	Haneda *et al*. (2012); Neumann *et al*. (2014)
SpvD	1/2	Inhibits nuclear transport of NF-κB p65	Rolhion *et al*. (2016)
SseF	2	Inhibits dendritic cell migration	McLaughlin *et al*. (2014)
SseI/ SrfH	2	Modulates cell migration of macrophages and dendritic cells	McLaughlin *et al*. (2009), Worley *et al*. (2006)
SseK1	1/2	Interferes with NF-κB activation	Li *et al*. (2013)
SseK3	2	Interferes with NF-κB activation	Yang *et al*. (2015)
SseL	2	Inhibits autophagy	Mesquita *et al*. (2012)
SspH1	1/2	Inhibits NF-κB-dependent gene expression	Haraga and Miller (2003), Haraga *et al*. (2006)
SspH2	2	Enhance SG1-dependent NLR-mediated immunity	Bhavsar *et al*. (2013), McLaughlin *et al*. (2014)
		Inhibits dendritic cell migration	
SteA	1/2	Represses genes involved in immune responses	Cardenal-Muñoz *et al*. (2014)
SteB		Biofilm formation	Dong *et al*. (2008), McGhie *et al*. (2009)

develop the disease. Their observations also indicate that *S.* Typhi-specific $CD8^+$ T migrated not only to mucosal sites, but also to secondary lymphoid tissues. These localized events, delay disease onset. They also suggested that co-production of MIP-1β with other cytokines is a key component in protection against *S.* Typhi (Fresnay *et al.*, 2016). This type of studies is key to identify an early selection of novel vaccine candidates for further evaluation in clinical trials. These models are also necessary to understand the role of certain T3SS effectors that may only be relevant in certain cell types or animal species.

Advances in techniques for single cell analysis allow us to study bacterial subpopulations that have different expression profiles of effectors and other virulence factors.

Finally, a more precise understanding of the interactions between *Salmonella* and the host immune system could help us design new approaches to modify the progression of *Salmonella* infections, strengthening key components of the immune response.

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