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Innate immune response to *Salmonella typhimurium*, a model enteric pathogen

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The innate immune system provides the first line of defense against invading microorganisms by inducing a variety of inflammatory and antimicrobial responses. These responses are particularly important in the gastrointestinal tract, where the needs for efficient nutrient uptake and host defense collide. Many pathogens have evolved to specifically colonize the intestine, causing millions of cases of enteric infections a year. A paradigm of an enteric pathogen is *Salmonella enterica*, a gram-negative bacterium that causes a wide range of gastrointestinal and systemic diseases. Infections with *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) lead to an acute intestinal inflammation in human and animal hosts, as a result of the bacterium invading the mucosa. A distinctive feature of Salmonella is that it has not only adapted to survive in a strong inflammatory environment, but it also uses this adaptation as a strategy to gain a growth advantage over the intestinal microbiota. We will use the model organism *S. typhimurium* to discuss the innate immune mechanisms employed by the mammalian gastrointestinal system and how the pathogen responds and subverts these mechanisms. In particular, we focus on the recognition of extra- and intra-cellular Salmonellae by germline-encoded pattern recognition receptors of the TLR and NLR families, and how Salmonella might profit from the activation of these receptors.

Innate Immunity

The innate immune system comprises many different mechanisms and components that act in a coordinated fashion to restrict an infection and eliminate the invading pathogen. A diverse array of immune cells cooperate in the rapid recognition and elimination of invading microbes through phagocytosis-mediated killing and the induction of inflammation.[1] Acute inflammation is characterized by an increased blood flow, rapid influx and activation of immune cells, and the release of pro-inflammatory cytokines. This results in heat, swelling and redness of the affected area and serves to prevent the spread of the infection and to promote pathogen clearance and tissue repair. The inflammatory response needs to be tightly regulated and quickly terminated to prevent unnecessary damage to tissues and to the organism.

Over the past decade, compelling evidence has demonstrated that detection of invading microbes is based on germline-encoded pattern-recognition receptors (PRRs), which recognize conserved microbial molecules, called pathogen- associated molecular patterns (PAMPs).[2] In addition to PAMPs, PRRs also respond to endogenous, host-derived danger signals, termed DAMPs (Danger-associated molecular patterns) or alarmins, which are released in response to tissue injury, stress or necrotic cell death. Several families of pattern-recognition receptors have been discovered thus far and are known to engage in the surveillance of both the extracellular and intracellular space. These families include the membrane-bound Toll-like receptors (TLRs) and C-type lectins that recognize PAMPs/DAMPs in the extracellular compartment and in endosomes, and recognize the cytosolic NOD-like receptors (NLRs), PYHIN-proteins and RIG-I-like receptors (RLRs) that monitor the intracellular space. These receptors allow the discrimination of various types of tissue insults and launch appropriate inflammatory responses.

The Gastrointestinal Immune System

The immune system is particularly important in the gastrointestinal (GI) tract, since it is the location where the divergent needs of nutrient absorption and host defense collide. The former requires a large surface area and a thin epithelium, which in turn has the potential to compromise host defenses. It is therefore not surprising that many pathogens have evolved to take advantage of this apparent impairment. The importance of protecting the gut from these pathogens is evident in the abundance of lymphoid tissue and immune cells it harbors. Yet, the most difficult challenge that the gastrointestinal immune system has to face is to respond to pathogens while remaining relatively unresponsive to food antigens and the commensal microflora. The need for tight control of the inflammatory response and the maintenance of gut homeostasis is highlighted by the severity of chronic auto-inflammatory bowel diseases, such as Crohn disease and ulcerative colitis (UC).[3]

The primary cellular barrier of the gut is a single layer of intestinal epithelial cells, which are critical for nutrient uptake yet provide a physical and chemical barrier (**Fig. 1**). This gastrointestinal barrier not only blocks the entry of commensals and

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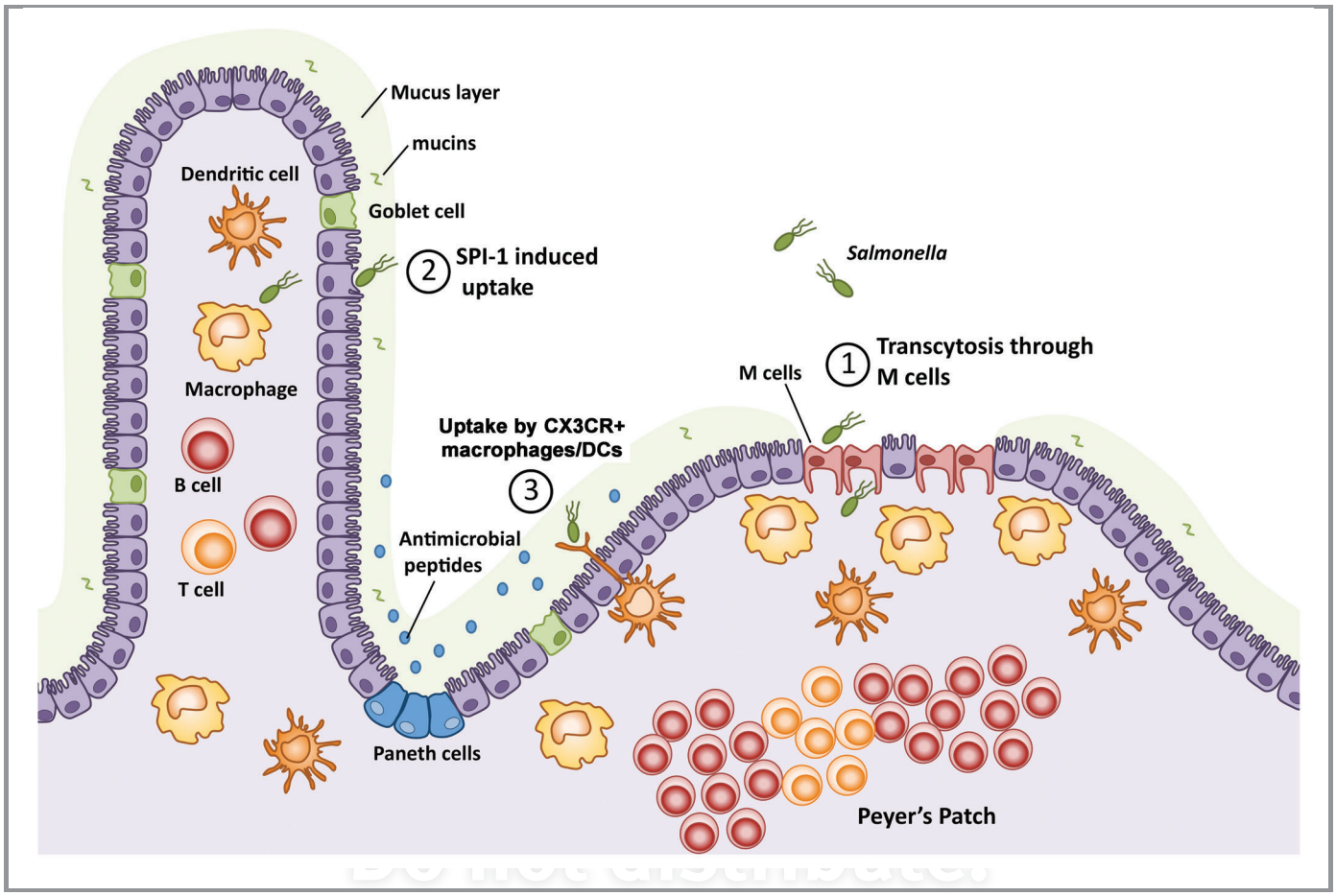

Figure 1. Schematic representation showing the different routes Salmonella can take in order to invade the intestinal mucosa. (1) The major pathway of invasion is through M cell mediated transcytosis at the Peyer's patches. (2) An alternative route is via uptake by enterocytes. (3) This route requires the injection of bacterial effector proteins by the SPI-1 Type 3 Secretion System that induce ruffling and uptake of the bacterium. Uptake through intercalating CX3CR1^{+} macrophages/DCs might represent an additional route of invasion.

pathogens, but also normally prevents the innate immune system from encountering commensal-derived antigens. Each epithelial cell maintains intimate association with its neighbors and seals the surface of the gut with tight junctions. Non-hematopoietic cells in the epithelial layer help to maintain this barrier by secreting mucus (goblet cells) or antimicrobial peptides (Paneth cells) (**Fig. 1**). Underlying this epithelial cell layer is the lamina propria, which contains a highly organized lymphoid tissue commonly referred to as gut-associated lymphoid tissue (GALT). The GALT is comprised of isolated and aggregated lymphoid follicles and is populated by a wide array of lymphocytes, such as T cells and B cells, dendritic cells, macrophages and neutrophils (**Fig. 1**). These cells regulate inflammatory responses to bacteria and antigens that breach the gastrointestinal barrier, protect the mucosa against harmful pathogens, and scavenge dead cells and foreign debris. Aggregated lymphoid follicles, so-called Peyer's patches (PP), are surrounded by a particular epithelium, the follicle-associated epithelium (FAE), which forms the interface between the GALT and the luminal microenvironment. The PPs have an important role in the immune surveillance of the intestinal lumen and facilitate the induction of defense against pathogens or immune tolerance (oral tolerance) as a result of the complex interplay between immune cells located in the lymphoid follicles and the FAE. The FAE contains specialized cells named M (for microfold) cells, which transport luminal antigens and bacteria to the basolateral side by transcytosis. At their basal surface, the cell membrane of M cells is extensively folded around underlying lymphocytes and antigen-presenting cells that activate or inhibit the immune response leading to either tolerance or systemic immune responses. In addition, antigen-presenting dendritic cells (DCs) send processes between intestinal epithelial cells without disturbing tight junction integrity and sample antigens from commensal and pathogenic gut bacteria. B cells and memory cells get activated by antigen-presenting cells in PPs and then travel to the mesenteric lymph nodes (MLN), where the immune response is amplified. Thus the GALT plays a crucial part in the interplay between the innate and adaptive immune system.

Salmonella Infections

Salmonella enterica is a flagellated, Gram-negative, facultative intracellular bacterial species that is a leading cause of enteric

disease in humans and in animal hosts. Salmonellae are taken up via contaminated food and can infect a broad range of hosts; certain host-adapted serovars can even cause a severe systemic disease. For example, the human restricted *S. enterica* serovar Typhi causes Typhoid fever, which affects over 20 million people worldwide and leads to approximately 200,000 deaths per year. In contrast, *S. enterica* serovar Typhimurium (*S. typhimurium*) causes a self-limiting gastroenteritis in humans, while mice infected with this serovar get a systemic disease with a pathogenesis resembling typhoid fever in humans. Thus murine infections with *S. typhimurium* are studied as a model for human Typhoid infections. Interestingly the disease outcome varies significantly between different inbred mouse strains. C57BL/6 mice get an acute infection leading to death within 6–8 d post-infection; 129Sv mice contain the infection and can become persistently infected,[4] which is reminiscent of *S. typhi* infections. Humans with Typhoid can become persistently infected once the initial acute phase has subsided, and this population serves as a significant reservoir for disease transmission.

After being ingested with contaminated food, Salmonellae reach the gut lumen where they have to compete with the resident intestinal microbiota for nutrients. Colonization resistance is provided by the indigenous commensals and is an important but often overlooked barrier to infection with enteric pathogens. Treatment with an antibiotic such as streptomycin before the infection has been shown to reduce the infectious dose of *S. typhimurium* in mice by $>$ 100,000-fold and is therefore commonly used in some mouse models of Salmonella infection to circumvent colonization resistance.[5] Importantly, several groups have demonstrated that *S. typhimurium* uses its virulence factors to induce intestinal inflammation during the intestinal phase to gain a growth advantage over the microbiota. For example, reactive oxygen species generated during inflammation react with endogenous, luminal sulfur compounds (thiosulphate) to form a new respiratory electron acceptor, tetrathionate, which *S. typhimurium* has evolved to use, while commensals lack the required metabolic pathways.[6]

There are two main components to Salmonella infections in the intestine: adherence and subsequent invasion.[7] Several adhesins and fimbriae are necessary to mediate adherence to epithelial cells in the gut.[8] Following adherence, Salmonella can use several different mechanisms to cross the intestinal epithelium. (**Fig. 1**.) A major route of entry is through M cells in the FAE overlying PPs, which transport antigens and bacteria from the lumen to the lamina propria.[9] Salmonella can also induce its internalization in non-phagocytic enterocytes through its virulence-associated type 3 secretion system encoded by Salmonella pathogenicity Island 1 (SPI-1).[10] Invasion also has been proposed to occur by paracellular pathways following disruption of tight junctions[11] or via CX3CR1$^+$ macrophages/DCs, which intercalate between epithelial cells.[12-14] However, the importance of these invasion-independent alternative pathways remains to be determined.

After crossing the intestinal barrier at the site of PPs, the bacteria are taken up by phagocytic immune cells like macrophages. Once phagocytosed, Salmonellae replicate within a vacuolar compartment in the cytoplasm.[7] Crucial for this intracellular phase is a second type 3 secretion system encoded by Salmonella pathogenicity Island 2 (SPI-2). While non-typhoidal strains remain restricted to the GI tract, typhoidal Salmonella serovars then disseminate from the GI tract to mesenteric lymph nodes and colonize systemic sites, like the liver and spleen.

Innate Immune Response to Salmonella

Salmonella is a very successful enteric pathogen because it has developed strategies to cope with most of the immune defenses employed by the host during the different phases of the disease. In the following we will describe in detail the innate immune mechanisms employed by the host and how Salmonella reacts to and subverts these immune responses.

Gastric innate immune mechanisms. One of the first obstacles faced by Salmonella is a thick layer of mucous that covers the surface of the gut epithelium and provides an initial barrier, which must be penetrated in order to gain direct contact with the epithelium. This mucus layer is formed by mucins, a family of glycoproteins secreted by specialized types of epithelial cells, called Goblet cells (**Fig. 1**). In addition to the mucins, cells in the gastrointestinal tract secrete several types of antimicrobial peptides, which are small amphipathic proteins that function like peptide antibiotics by disrupting the integrity of the bacterial cell membrane. The production and release of antimicrobial peptides and mucins by the epithelial cells of the gut represents a major barrier against microbial invasion and an important part of the innate immune response. While some antimicrobial peptides are expressed constitutively, many are induced as part of the inflammatory response to invading pathogens.[15] Paneth cells of the intestinal crypts are the most abundant producers of constitutively expressed antimicrobial peptides in mammals, such as α- and β-defensins (cryptdins in mice), CRS peptides, lysozyme and phospholipase A2 (sPLA2).[16] They can be induced to secrete additional types of antimicrobial peptides, such as cathelicidins, angiogenin 4 (ANG4) and the C-type lectins RegIIIβ/γ[15], in response to microbial PAMPs. In addition to Paneth cells, epithelial cells are also known to produce inducible antimicrobial peptides such as RELM-b, RegIIIγ, calprotectin and β-defensins.

During *S. typhimurium* infections of Streptomycin-pretreated mice, RegIIIβ and RegIIIγ expression is markedly increased.[17] RegIIIγ secretion contributes to the clearance of other intestinal pathogens such as *Listeria monocytogenes* and Enterococcus spp.[18] However, unlike these pathogens Salmonella is better adapted to counter host defenses and might even profit from increased production of C-type lectins to gain a growth advantage over the microbiota. In vitro, RegIIIβ kills diverse commensal gut bacteria but not Salmonella, which can be attributed to its specific cell envelope structure.[19] RegIIIγ production is absent in mice deficient for MyD88, an adaptor of TLR signaling.[20] In addition to TLRs, cytokines of the IL-23/IL-22 axis (see below) might also be involved in the production of antimicrobial peptides (**Fig. 2**). In vitro, IL-22 is known to induce the expression of inducible Nitric Oxide Synthase (iNOS), the mucin MUC4 and the siderophore lipocalin-2 in human colonic epithelial cells.[21] In

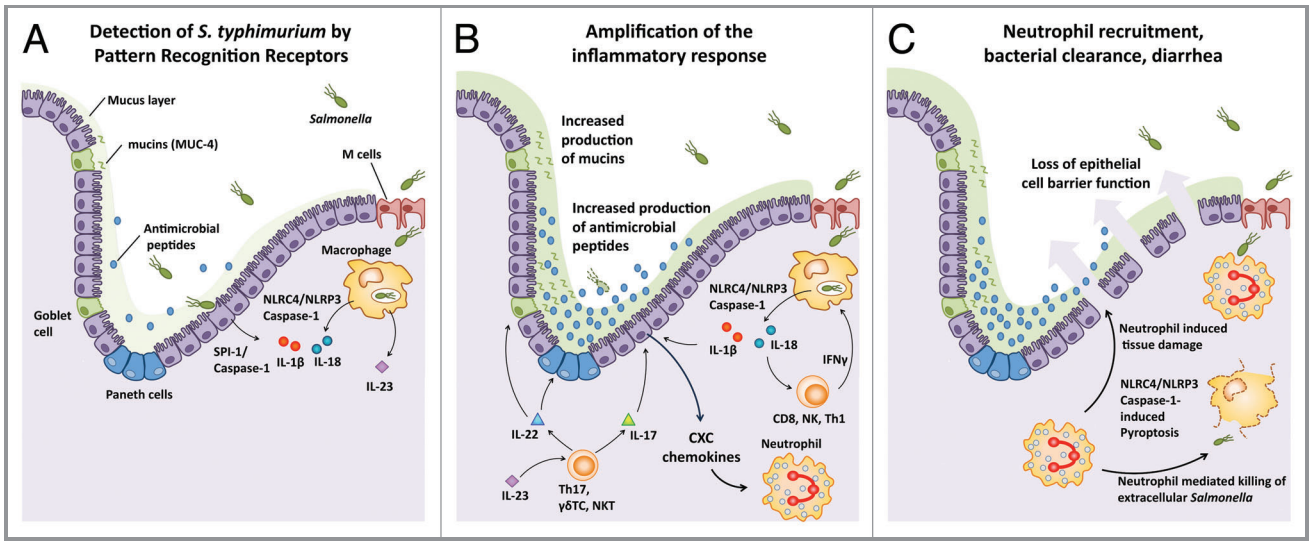

Figure 2. The gastric innate immune response to Salmonella. (A) Following the invasion of the mucosa, the presence of Salmonella is detected by pattern recognition receptors. Extracellular Salmonella are detected by Toll-like receptors inducing a transcriptional response leading to the expression of pro-inflammatory cytokines such as IL-23. Intracellular Salmonella activate NOD-like receptors that can induce IL-23 expression, as well as the assembly of NLRC4/NLRP3 inflammasomes that activate Caspase-1, promoting the secretion of mature IL-1β and IL-18. SPI-1 mediated activation of Caspase-1 in epithelial cells might contribute to IL-18 secretion. (B) IL-18 and IL-23 amplify the inflammatory response by paracrine signaling. IL-18 induces the release of IFNγ from T cells, while IL-23 induces the release of IL-22 and IL-17. These cytokines induce the increased production of mucins and antimicrobial peptides, and promote the release of CXC chemokines leading to an influx of neutrophils into the mucosa. (C) Infiltrating neutrophils are crucial for the killing of extracellular Salmonellae. Although considered an intracellular pathogen, Salmonella can be found extracellularly following transcytosis through M cells or after pyroptosis induced host cell lysis. Besides clearing the pathogen, neutrophil influx can also lead to damage to intestinal tissue, resulting in the loss of epithelial cell barrier function and promoting diarrhea.

vivo, lipocalin-2 is produced in response to *S. typhimurium* infections in the ileal mucosa of infected rhesus macaques. Expression of iNOS, calprotectin, MUC4, Duox2 and enteric β-defensins are all upregulated in the inflamed mucosa[22-24] (**Fig. 2**). Since *S. typhimurium* infection of cultured colonic epithelial cells does not induce the same response, it suggests that paracrine IL-22 signaling is required. Consistently during *Citrobacter rodentium* infections of mice, IL-22 is required for the expression of RegIIIγ and Calprotectin.[25]

In order to overcome the chemical barrier generated by these antimicrobial peptides, *S. typhimurium* has developed means to sense these host responses or effectors and to respond by upregulating corresponding virulence mechanisms. For example, the PhoP-PhoQ two-component signal transduction system, which Salmonella shares with many other bacteria, has been shown to sense the presence of cationic antimicrobial peptides as well as divalent cations and low pH.[26] Activation of PhoQ induces a transcriptional program resulting in increased resistance against antimicrobial peptides that is achieved by modifications of lipid A either by acylation (PagP) or addition of an amino-arabinose moiety (Pmr system). Salmonellae lacking PhoP or PhoQ have been shown to be severely attenuated in an in vivo model of infection and to confer partial protective immunity against subsequent wild-type Salmonella infections.[27] Additional resistance mechanisms to innate defenses in the gut include expression of enzymes encoded by the *iroBCDE iroN* locus, which alter the structure of the Salmonella Fe^{+} binding protein Ent, thereby preventing it from being bound and sequestered by lipocalin-2.[28] Indeed, Salmonella mutants lacking *iroN* fail to evade the scavaging effects of lipocalin-2 and are severely attenuated for growth in the gut.[21]

Detection of Salmonella by PRRs. Crossing the epithelial barrier allows Salmonella to escape the inhospitable environment at the surface of the intestinal mucosa and to evade many antimicrobial defenses. However, once Salmonella has passed through M cells or enterocytes, it encounters the next layer of innate immune defenses, the monocyte-derived phagocytic cells of the GALT: macrophages and dendritic cells. The main function of these cells is to remove invading microbes by phagocytosis and to alert other immune cells of the infection, either directly or by secreting pro-inflammatory cytokines. Following phagocytosis, Salmonellae express their virulence-associated SPI-2 T3SS to establish themselves in an intracellular compartment named the Salmonella containing vacuole (SCV).[7] Within this compartment Salmonella can replicate to high numbers before exiting the cell and infecting new host cells. Although Salmonella remains partially hidden within its intracellular niche, it cannot completely escape host cell sensing. All monocytic cells express an array of germline-encoded pattern recognition receptors (PRR), which enable them to detect pathogen-associated molecular pattern molecules (PAMPs). Toll-like receptors (TLRs), which are located on the outer membrane of the cell (TLR1, 2, 4, 5, 6, 10) or in intracellular vesicles (TLR3, 7, 8, 9, 11, 13 early and late endosome, lysosomes), are the first PRRs to detect the presence of Salmonella.[29] TLRs can detect a variety of extracellular and endosomal PAMPs such as LPS, bacterial lipoproteins,

peptidoglycan, flagellin DNA, RNA and others. Upon ligand binding, TLRs engage the signaling adaptors MyD88 and TRIF, which initiate signaling cascades leading to the activation of the transcriptional factors NFκB and IRF3 that induce the production of inflammatory cytokines (IL-8, IL-10, pro-IL-1β, pro-IL18 and others) as well as a type I IFN response, respectively.[29]

In vitro, a multitude of TLRs can be activated by Salmonella-derived ligands, such as lipoproteins (TLR1/2/6), LPS (TLR4), the Salmonella flagellin FliC (TLR5) and CpG-rich repetitive elements in Salmonella DNA (TLR9). In addition, TLR2 is known to recognize CsgA, a subunit of the amyloid curly fibers that form the biofilm matrix of *S. typhimurium*.[30] TLR4 and TLR2 also play an important role in vivo since *tlr4*$^{-/-}$, *tlr2*$^{-/-}$ and *tlr4*$^{-/-}$/*tlr2*$^{-/-}$ mice have increased bacterial burdens in mesenteric lymph nodes (MLNs) following oral infection.[31] Intriguingly, Salmonella virulence seems to actually require TLR sensing. It was recently shown that *tlr4*$^{-/-}$/*tlr2*$^{-/-}$ mice are sensitive to Salmonella infection, but mice deficient in TLR2, TLR4 and TLR9 are less sensitive.[32] This can be attributed to the fact that the SCV in macrophages derived from *tlr4*$^{-/-}$/*tlr2*$^{-/-}$/*tlr9*$^{-/-}$ mice fails to acidify. Since acidification is an important indicator for Salmonella to sense the intra-phagosomal environment, Salmonella does not induce the expression SPI-2 virulence genes in *tlr4*$^{-/-}$/*tlr2*$^{-/-}$/*tlr9*$^{-/-}$ *cells.* Thus, although TLRs are clearly essential for defense against pathogens, Salmonella has evolved virulence mechanisms that are activated in response.

Salmonella flagellin is another PAMP sensed by PRRs. In the gut, extracellular Salmonella flagellin is detected by enterocytes expressing TLR5 on their basolateral surface, leading to NFκB activation and a robust IL-8 inflammatory response.[33] Although Salmonella has mechanisms to dampen this NFκB response, a recent study revealed that enterocytes might have the ability to bypass this effect by transmitting signals to neighboring uninfected cells to promote NFκB-mediated IL-8 release in response to Salmonella infection.[34]

Once Salmonella has established itself within the SCV, it is hidden from many extracellular detection mechanisms. Nevertheless, macrophages and other lymphocytes have evolved mechanisms to recognize the presence of PAMPs in the cytosol.[35] The NOD-like receptor (NLR) family of PRR is a surveillance system that can detect the presence of PAMPs in the cytosol. NLRs contain an N-terminal protein-protein interaction domain [either a Caspase Recruitment Domain (CARD) or a Pyrin-like domain (PYD)], a central nucleotide oligomerization domain (NOD), and a C-terminal Leucine-rich repeat domain (LRR).[35] Upon ligand binding, NLRs multimerize and initiate different signaling cascades. For example, NOD1 and NOD2 interact with RIP2 kinase, which is a potent activator of NFκB. The contribution of NOD1/2 signaling to the innate immune defense against Salmonella was recently shown by Geddes et al.[36] Mice deficient in NOD1/2 or RIP2 showed attenuated inflammatory pathology, reduced levels of inflammatory cytokines, and increased colonization of the mucosal tissue. Interestingly, bone-marrow chimeras demonstrated that this was dependent on NOD1/2 signaling in hemopoietic as well as non-hemapoietic compartments.

In addition to NODs, there is a family of NLRs that do not initiate a transcriptional program, but induce the assembly of a large multiprotein signaling complex, called the inflammasome.[37] Assembly of the complex usually also requires an adaptor protein called ASC (apoptosis-associated speck-like protein with a CARD), which recruits the cysteine protease pro-Caspase-1 to the inflammasome.[35] Within this complex pro-Caspase-1 is activated by dimerization and autoproteolytic cleavage.[38] Activated Caspase-1 cleaves the pro-forms of interleukin (IL)-1β and IL-18, leading to production and secretion of the mature cytokines. Caspase-1 activation also initiates a pro-inflammatory cell-death program called pyroptosis. Pyroptosis is named for "pyro" meaning fire and "ptosis" meaning to fall in Greek, which reflects its hallmark inflammatory outcome.[39] Pyroptosis is similar to apoptosis in that it is a programmed form of cell death controlled by caspases. Pyroptosis is solely dependent on Caspase-1. In contrast to apoptosis, pyroptosis is not a silent form of cell death. During pyroptosis, there is formation of pores in the cell membrane and subsequent release of cellular contents and pro-inflammatory cytokines, which serve to amplify the inflammatory response by signaling the recruitment of other mediators of inflammation. Inflammasome-induced pyroptosis likely benefits the host during microbial infections by eliminating the intracellular niche of the pathogen and re-exposing it to extracellular immune defenses.[40]

S. typhimurium infections of bone marrow-derived macrophages in tissue culture rapidly activates NLRC4, a cytoplasmic flagellin sensor, that initiates inflammasome assembly and Caspase-1 activation.[41] The activation of the NLRC4 inflammasome requires the expression of the SPI-1 T3SS and flagellin. These results led to the hypothesis that the SPI-1 needle complex, which is evolutionarily related to the flagellar apparatus, was accidentally translocating flagellin into the macrophage cytosol.[42,43] However, Salmonellae deficient in flagellin are still capable of inducing a NLRC4 inflammasome response, suggesting that there was an additional NLRC4 ligand produced by Salmonella. Subsequent studies revealed that PrgJ, the needle complex rod protein, was also capable of stimulating the NLRC4 inflammasome.[44]

Activated Caspase-1 is a very important mediator of the host response to Salmonella. Mice lacking Caspase-1 are much more sensitive to Salmonella infection and have higher bacterial burdens in systemic organs compared with wild-type mice.[45,46] This is due at least in part to the production of IL-1β and IL-18, since mice lacking either cytokine are also more susceptible to Salmonella infection than wild-type mice.[45] However, NLRC4-deficient mice harbored comparable bacterial loads as wild-type mice following oral infections with wild-type Salmonella.[46] The discrepancy between the in vitro and in vivo findings was recently explained by our results showing that Salmonellae can also activate Caspase-1 through NLRP3, another cytosolic receptor of the NLR protein family.[47] Salmonella grown to stationary phase, which results in the downregulation of the SPI-1 T3SS and the expression of the SPI-2 T3SS, activated two NLRs simultaneously: (1) NLRC4-dependent cytokine release was stimulated by SPI-2 T3SS-dependent translocation of flagellin. (2) NLRP3-dependent

cytokine release is stimulated by an unknown ligand.[47] Consistent with these results, NLRP3 and NLRC4 were shown to play redundant roles in vivo since mice deficient in both NLRP3 and NLRC4 had higher bacterial loads in MLN, spleen and liver, similar to Caspase-1-deficient mice, while mice deficient in only one NLR were comparable to wild-type mice.[47]

Although in vitro infection models have established that Salmonella flagellin is a potent activator of innate immune sensors such as TLR5 and NLRC4, the importance of this recognition for the different phases of a Salmonella infection remains unclear because Salmonellae downregulate flagellin expression in vivo.[48,49] For example, Cummings et al. showed that FliC expression was anatomically restricted in mice infected orally with *S. typhimurium*, and *fliC* is expressed in the PP, but not in the mesenteric lymph nodes and spleen.[49] In addition, flagellin-deficient Salmonellae have been shown to have comparable virulence to wild-type bacteria.[50,51] This downregulation of flagellin expression appears to be an important virulence mechanism, since the growth of Salmonellae constitutively overexpressing flagellin are highly attenuated in a mouse model of infection.[52] Growth restriction is most likely mediated by the NLRC4-Caspase-1 axis, since in contrast to wild-type Salmonella, Salmonella overexpressing flagellin are detected by NLRC4, a PRR that induces the assembly of Caspase-1 activating inflammasome complexes in the presence of flagellin in the cytoplasm.[40] Activation of Caspase-1 was suggested to induce pyroptosis of infected host macrophages, release of the pathogen and subsequent neutrophil mediated clearance.[40] Since NLRC4 plays a redundant role with NLRP3 in activating Caspase-1 in vivo, flagellin sensing by NLRC4 is likely to be important for host defense early in the infection, most likely in the GI tract where the bacteria still express flagellin. However, during the systemic phase, NLRP3 recognizes intracellular Salmonellae and mediates inflammasome-dependent defense.

Mucosal inflammation during *S. typhimurium* infections. A hallmark of *S. typhimurium* infections is an acute, mucosal inflammation in the gut. This inflammation is a stereotypic host response triggered by the detection of bacterial PAMPs within the mucosa by PRRs and is induced in response to Salmonella penetrating the intestinal epithelium and surviving in tissue macrophages. Invasion and intracellular replication requires the coordinated action of the two virulence-associated T3SSs called SPI-1 and SPI-2. Consistently, both SPI-1 and SPI-2 were shown to contribute to intestinal inflammation after oral infections of calves or streptomycin pretreated mice.[53-55] In vitro infections of cultured macrophages led to the identification of a variety of mechanisms by which the host can recognize the presence of *S. typhimurium* (see above). Activation of these PRRs leads to the expression and secretion of key cytokines such as IL-18 and IL-23 (**Fig. 2A**), which amplify the inflammatory response by paracrine signaling mechanisms, inducing the massive secretion of IFNγ, IL-22 and IL-17 by mucosa-resident T cells (**Fig. 2B**). In particular, IL-18, which is released in an inflammasome dependent manner, is critical to induce T cells to secreted IFNγ release in vivo.[56] Consistently, Caspase-1-deficient mice are deficient for gamma interferon expression in the murine cecum early (12 h) after *S. typhimurium* infection.[57] Interestingly, IL-18 release in the mucosa is independent of flagellin recognition since flagellin-deficient *S. typhimurium* induce comparable levels of IL-18 and IFNγ [57]. The nature of the ligand and the receptor inducing Caspase-1 activation remains unknown thus far. Another major inflammatory pathway in the mucosa is controlled by the release of IL-23. The levels of IL-17 and IL-22 increase dramatically within 2–5 h post-infection in bovine or avian ligated loops.[22,23] Such an early amplification of host inflammatory responses could involve the stimulation of antigen-experienced T cells by IL-23, inducing the release of IL-17 and IL-22. Consistently, *IL-23*$^{-/-}$ mice do not produce IL-17 and IL-22 in response to *S. typhimurium* infections.[17] In vitro, IL-23 is mainly produced by macrophages and dendritic cells, yet its source during *S. typhimurium* infections remains unknown. In addition to T cells, Innate Lymphoid Cells (ILCs), which include natural killer cells (NK cells) and lymphoid tissue-inducer cells (LTi cells), could be potential sources of IL-22.[58,59] In particular CD4$^+$ LTi cells play a critical role in the development of lymphoid tissues and in inflammatory responses. CD4$^+$ LTi cells have recently been shown to be a major source of IL-22 production in response to infections with *C. rodentium*.[60] This response was dependent on IL-23 and was induced in response to Lymphoid toxin (LT), a member of the Tumor Necrosis factor (TNF) core family that is expressed mainly by lymphocytes, including T, B, NK and LTi cells.[61,62] However, a role of LTi cells in *S. typhimurium* infections remains to be demonstrated. Distinct T cell populations express the IL-23 receptor on their surface, including Th17 cells, γδT cells and NK T cells.[17,63-65] The combination of all of these cytokines induces a strong inflammatory environment in the intestine, which is characterized by an increased production of anti-microbial peptides (IL-22/23 axis) and a recruitment of neutrophils by the IL-17/23 axis, leading to diarrhea and the deprivation of nutrients, which affects commensals as well as the pathogen (**Fig. 2C**).

Neutrophil influx during *S. typhimurium* infections. The activation of innate immune responses in the mucosa and the resulting inflammation serves as an activator of further innate immune responses. One of these is the recruitment of neutrophils to the mucosa, which is mostly mediated via the IL-23/IL-17 axis (**Fig. 2**). The production of IL-17 stimulates the secretion of CXC chemokines by intestinal epithelial cells and granulopoiesis in the bone marrow by inducing the production of G-CSF (granulocyte colony-stimulating factor).[66] Consistently, IL-17-deficient mice have a severe defect in the recruitment of neutrophils to the intestinal mucosa.[22] IL-1β production could also contribute to neutrophil recruitment, since Caspase-1-deficient mice exhibit reduced levels of CXC chemokines in the cecal mucosa early after *S. typhimurium* infection.[57] The recruitment of neutrophils is crucial to prevent the dissemination of Salmonella from the gut, since neutropenia increases the risk of systemic infections.[67,68] Although *S. typhimurium* is mainly considered an intracellular pathogen, the role of neutrophils in containing the infection is likely to involve ingestion and killing of extracellular bacteria.[69] Salmonella is susceptible to neutrophil-mediated killing when it exits epithelial cells and transits to

phagocytes or when it spreads to new host cells (**Fig. 2C**). Consistent with this idea, it has been shown recently that Salmonellae constitutively expressing flagellin are released from host cells via NLRC4/Caspase-1-induced pyroptosis and are subsequently removed by neutrophils.[40] In addition, Salmonella replicate extracellularly after depletion of neutrophils.[70] Although neutrophils are important for host defense against Salmonella, infiltrating neutrophils are also a major cause of tissue damage in the mucosa, which is sometimes associated with necrosis in large areas of the terminal ileum and colon.[53] This tissue damage leads to a loss of epithelial barrier function, resulting in an increase in inflammation and contributing to diarrhea. In addition, neutrophils might contribute to diarrhea by stimulating chloride secretion from epithelial cells.[71] Thus, although neutrophil recruitment prevents the systemic spread of the infection, it might nevertheless be a key determinant for the development of gastroenteritis.

Active induction of inflammation by *S. typhimurium*. Although *S. typhimurium* cannot resist the onslaught of neutrophils in intestinal tissue, the pathogen appears to be custom-built to bloom in the lumen of the inflamed intestine, because its numbers in this niche increase dramatically during inflammation. Above, we have discussed the different mechanisms Salmonella has evolved to gain this advantage allowing it to survive and replicate in the inflamed gut despite diarrhea and nutrient restriction.[72] Considering the significant growth advantage that Salmonella gains from intestinal inflammation, it would not be surprising if Salmonella would actively induce host innate immune pathways to cause inflammation. Indeed, the W.D. Hardt and J.E. Galan research groups have recently published studies that support such a mechanism.[73,74] Bruno et al. report that infections of human epithelial cells induced a transcriptional response through the activation of mitogen-activated protein (MAP) kinase and NFκB signaling.[73] This activation was dependent on the SPI-1 bacterial effector proteins SopE, SopE2 and SopB, and partially on the activation of Rho-family GTPase Cdc42, but independent of intracellular sensors such as NOD1, NOD2 and RIP2 kinase. In addition, *S. typhimurium* induced intestinal inflammation in mice deficient for TLR4 and MyD88, consistent with an active role of SPI-1 effectors in the activation of innate immune responses.[73] In addition to a role in the activation of NFκB signaling, SopE seems to also have a role in the activation of Caspase-1.[74] Mueller et al. report that infection of HeLa cells or RAW264 macrophages with wild-type *S. typhimurium* or with a strain that expresses SopE and not the other SPI-1 effectors induced the activation of Caspase-1, as measured with the fluorescent Caspase-1 activity probe FLICA, and IL-1β secretion.[74] This activation was independent of flagellin, a known activator of the NLRC4/Caspase-1 axis.[75] Consistent with the in vitro findings, the effector-less mutant expressing SopE induced intestinal inflammation in wild-type, but not in Caspase-1-deficient mice. Similarly this strain failed to induce inflammation in *il-1R*$^{-/-}$ and *il-18*$^{-/-}$ mice, again supporting an important role of IL-18 in mucosal inflammation. Finally, bone-marrow chimeras demonstrated that inflammation induced by this effector-less mutant strain expressing SopE was dependent on Caspase-1 expression in stromal but not lymphoid cells, suggesting that SopE-induced Caspase-1 activation happened in gut epithelial cells and not mucosa resident lymphocytes. Although these results suggest that T3SS effector proteins could possibly activate Caspase-1 directly, the significance of these results for infections with wild-type Salmonella is still unclear. The effector-less mutant strain expressing SopE should be compared with wild-type bacteria or to a SopE single deletion mutant in the same analyses. In addition, several wild-type strains of Salmonella lack SopE, yet are still able to induce activation of Caspase-1. However, the notion that Caspase-1 could also be active in stromal cells in the gut was recently strengthened by the discovery of the NLRP6 inflammasome.[76] Unlike other NLRs, NLRP6 is highly expressed in the epithelial cells of the intestine, while its expression is low in lymphoid cells. NLRP6 seems to be involved in gut homeostasis through Caspase-1 mediated IL-18 secretion.[76] The ligand activating NLRP6 remains undiscovered so far, but it is hypothesized that it could be a bacterial PAMP indicating epithelial cell integrity. Interestingly, IL-18 is an important trigger of intestinal inflammation during Salmonella infections, yet if Salmonellae activate NLRP6 in the gut and if they do this actively by its T3SSs remains to be determined.

Summary and Outlook

In recent years, there has been a rapid increase in our understanding and appreciation of the importance, function and mechanisms of the innate immune system. This includes establishing the molecular basis for the detection of extra- and intra-cellular pathogens by germline-encoded, pattern-recognition receptors. These receptors play a crucial role in the GI tract in recognizing the presence of invading pathogens and inducing mucosal inflammation. Investigating Salmonella pathogenesis has significantly contributed to our understanding of how pathogens are detected in the gut and also how they respond to innate immune defense mechanisms. A particularly interesting field of study is how Salmonella has adapted to thrive in the gut despite massive inflammation, illustrating how a pathogen has evolved to trigger intestinal inflammation in order to outcompete the intestinal microbiota. Recent reports have revealed fascinating insights into how Salmonella takes advantage of the immune responses in the mucosa and turns them in its favor. For example, inflammation helps Salmonella to outcompete the microbiota by ROS generating a novel respiratory electron acceptor, which can be used by Salmonella but not the microbiota. In addition, the detection of Salmonella by TLRs has been shown to be crucial for Salmonella virulence, since it induces the acidification of the phagosome, which in turn provides a cue for Salmonella that it has reached its intracellular niche protected from extracellular immune responses.[32] Finally, recent reports indicate that Salmonella might have evolved the ability to actively trigger NFκB and inflammasome signaling pathways through injected effector proteins.[74]

Despite these recent advances, we are only beginning to decipher the role of different bacterial ligands, host pattern recognition receptors and different cell types in directing the

innate immune response to Salmonella. The recent discovery of NLRs and the inflammasome has significantly expanded the repertoire of possible innate immune detection mechanisms, yet the function of most known NLRs remains undiscovered. In addition, it has become clear that different cell types express a distinct set of PRRs and might trigger a different inflammatory program in response to pathogen infection. Finally, future studies will also need to address how the different innate immune responses shape adaptive immune responses and how this influences Salmonella pathogenesis.

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