Innate Immune Detection of Flagellin Positively and Negatively Regulates Salmonella Infection

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Abstract

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Salmonella enterica serovar Typhimurium is a flagellated bacterium and one of the leading causes of gastroenteritis in humans. Bacterial flagellin is required for motility and also a prime target of the innate immune system. Innate immune recognition of flagellin is mediated by at least two independent pathways, TLR5 and Naip5-Naip6/NlrC4/Caspase-1. The functional significance of each of the two independent flagellin recognition systems for host defense against wild type *Salmonella* infection is complex, and innate immune detection of flagellin contributes to both protection and susceptibility. We hypothesized that efficient modulation of flagellin expression *in vivo* permits *Salmonella* to evade innate immune detection and limit the functional role of flagellin-specific host innate defenses. To test this hypothesis, we used *Salmonella* deficient in the anti-sigma factor

flgM, which overproduce flagella and are attenuated *in vivo*. In this study we demonstrate that flagellin recognition by the innate immune system is responsible for the attenuation of *flgM- S.* Typhimurium, and dissect the contribution of each flagellin recognition pathway to bacterial clearance and inflammation. We demonstrate that caspase-1 controls mucosal and systemic infection of *flgM- S.* Typhimurium, and also limits intestinal inflammation and injury. In contrast, TLR5 paradoxically promotes bacterial colonization in the cecum and systemic infection, but attenuates intestinal inflammation. Our results indicate that *Salmonella* evasion of caspase-1 dependent flagellin recognition is critical for establishing infection and that evasion of TLR5 and caspase-1 dependent flagellin recognition helps *Salmonella* induce intestinal inflammation and establish a niche in the inflamed gut.

We also hypothesized that the Naip5/Nlrc4 inflammasome has distinct roles during systemic and mucosal detection of flagellin. To test this hypothesis, we used wildtype, flagellin deficient and flagellin overproducing *Salmonella* to assess the contribution of each inflammasome component during systemic and mucosal infection.

We demonstrate that during systemic infection, *Salmonella* efficiently evades flagellin detection. During mucosal infections, Casp1 and Nlrc4 are required for flagellin detection and host protection. In contrast, Naip5 has a more complex function. Similar to Nlrc4 and Casp1, loss of Naip5 abrogates *Salmonella* flagellin detection *in vivo*, and increases host susceptibility to infection. In addition, loss of Naip5 leads to protection against *Salmonella* infection, which is independent of flagellin expression by *Salmonella*. Thus Naip5 detection of *Salmonella*'s flagellin protects against infection, and Naip5 promotes *Salmonella* infection through an undefined mechanism, possibly involving interactions with the commensal microbiota. Our studies illuminate the complexity of mucosal infections, where the multitude of interactions between microbiota, pathogens and the innate immune system shape host defenses.

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Chapter 1: Introduction

The epidemiology of Salmonellosis

Salmonella is a common and widely distributed foodborne pathogen responsible for millions of human infections each year. According the World Health Organization (WHO), *Salmonella* is responsible for 94 million non-typhoidal *Salmonella* infections every year resulting in 155,000 deaths worldwide [1]. Most human cases of infection occur in the developing world with a strong prevalence in the Indian Subcontinent and South East Asia. However, the infection rate of *Salmonella* in the developing and third world is difficult to assess due to poor record keeping. Salmonellosis in humans is generally contracted via the oral route by consuming contaminated food of animal origin or vegetables exposed to manure. We only need to remember the recall of half a billion eggs in August 2010 to remember the constant threat that *Salmonella* poses to our food supply (Associated Press 20/Aug/2010). In this study we explored the immune responses against *Salmonella typhimurium*, a strain commonly associated with gastrointestinal infections. Most cases of Salmonellosis are characterized by acute onset of fever, abdominal pain, diarrhea and vomiting (Fig 1.1). As is usually the case with most diseases, the young, the elderly and the immunocompromised have a higher risk of mortality.

There are several factors that predispose to severe *Salmonella* infections i) HIV/AIDS infected patients are more vulnerable to

bacterial and viral infections and in the case of *Salmonella*, it is a major factor leading to bacteremia and life threatening Salmonellosis [2], ii) co-infection with malaria leads to increased *Salmonella* susceptibility often resulting in fatal bacteremia [3], iii) Multi-drug resistant *Salmonella* cases are emerging, challenging current treatments [4]. Most of the drug resistant strains of *Salmonella* have been discovered in Southeast Asia and Africa, regions that coincidentally have the highest rates of malaria infection, HIV infection and highest number of people living with HIV/AIDS (WHO). In addition, it has been hypothesized that the weak immune system and response of HIV/AIDS patients provides an appropriate niche for some strains of *Salmonella* (such as ST313) to evolve into multidrug resistant, immune evading hypervirulent strains that are capable of causing severe forms of infection to healthy adults [5,6].

Innate immune defense against Salmonella

Salmonella must overcome many obstacles imposed by the host in order to colonize and persist as a pathogen. The defenses against *Salmonella* at early stages of infection range from physiological barriers (i.e. acidity of the stomach or the intestinal epithelial layer) to specific immune cells actively protecting the host (i.e. neutrophils, macrophages, dendritic cells, and innate lymphoid cells). The clinical course of infection varies from asymptomatic cases to full blown systemic infection and sepsis. Human genetic studies performed on more than 150 individuals have found that defects on the IL12/IL23/IFN-γ axis correlate with recurrent severe *Salmonella*

infections suggesting that IFN-γ producing innate immune and T cells may be responsible for controlling non-typhoidal *Salmonella* infections in humans [7-11].

After ingestion *Salmonella* has to endure the gastric environment that has acidic pH and abundant nitric oxide that is produced as byproduct of dietary nitrate metabolism [12]. Once in the small intestine, *Salmonella* is challenged by i) mucus and ii) antimicrobial peptides. Goblet cells generate and secrete large amounts of mucus into the lumen of the intestine generating a physical barrier that prevents commensal and pathogenic bacteria from reaching intestinal epithelial cells. Paneth cells produce and secrete antimicrobial peptides to further strengthen the mucosal barrier and separate the gut microbiota from host cells [13]. An additional protective measure in the intestine is the commensal bacteria. Although not part of the host, a complex gut microbiota protects against pathogenic bacteria, possibly through direct competition for nutrients, production of anti-microbial factors and interactions with the host mucosal immune system [14].

Despite this impressive barrier *Salmonella* is capable of penetrating the mucus and breeching the intestinal epithelial barrier by three different mechanisms i) direct invasion of intestinal epithelial cells and subsequently crossing the intestinal barrier, ii) uptake by M cells located in the follicular dome epithelium over lymphoid patches in the small and large intestine, and iii) direct uptake by dendritic cell protrusions that extend between epithelial cells and reach out into the

lumen of the intestine [15-17]. Once *Salmonella* makes contact with host cells, innate immune receptors trigger the production of cytokines and other factors that recruit neutrophils and macrophages to phagocytose and eliminate the pathogen. The physiology of the gastro-intestinal tract and the ability innate immune cells to recognize conserved bacterial ligands create outstanding barriers. In order to overcome these barriers, *Salmonella* has two main pathogenicity islands (Spi1 and Spi2), which encode biological functions that are necessary to facilitate entry and survival within the host [15].

In this thesis, we will embark on a journey to understand how *Salmonella's* regulation of the protein required for bacterial motility, flagellin, leads to virulence and immune modulation.

Flagellin recognition

Innate immune recognition of *Salmonella* is mediated by evolutionarily conserved receptors that are capable of sensing conserved bacterial structures. These innate immune receptors, referred to as pattern recognition receptors (PRRs), recognize conserved pathogen associated molecular patterns (PAMPs) and regulate immune responses [18]. PRRs recognize a wide range of bacterial ligands such as bacterial DNA, cell wall components (peptidoglycan and LPS) and flagellin (Fig 1.2). In this study, we focus on flagellin recognition by the innate immune system. Bacterial flagellin has been studied for decades due to its importance in motility, and more recently for its ability to activate the innate immune

system. There are two known flagellin recognition pathways: i) an intracellular pathway mediated by Naip5/Nlrc4/Caspase 1 and ii) an extracellular pathway mediated by Toll-like receptor 5 (TLR5) (Fig 1.3).

i) Intracellular flagellin recognition pathway

A conserved site in the C-terminus of flagellin is sensed by the intracellular receptor Naip5 in macrophages, leading to the oligomerization of Nlrc4, activation of caspase 1 and secretion of potent proinflammatory cytokines [19-21]. Naip5 forms hetero-oligomers with Nlrc4, which interacts with and activates Caspase-1 via its caspase activating region domain (CARD) [22]. Recently published data using *in vitro* HEK293 reconstitution assays have demonstrated that intracellular flagellin, specifically the C-terminus, activates Naip5, leading to the subsequent recruitment of Nlrc4 to form Naip5-Nlrc4 hetero-oligomeric complexes [23]. Naip5-Nlrc4 complexes activate caspase-1 which leads to cell death and the release of processed IL-1B and IL-18 [24]. IL-1B and IL-18 perform several functions that include: 1) macrophage, dendritic cell and neutrophil activation 2) T helper cell differentiation, and 3) fever [25,26].

Unlike TLR5, which senses monomeric flagellin that is present in the extracellular space, the Naip5/Nlrc4/Caspase 1 pathway requires the translocation of flagellin into the cytosol of the macrophage. A wealth of data from *in vitro* studies supports a model where a bacterial type

III or type IV secretion system (T3SS), commonly used by pathogenic bacteria to inject effector molecules into host cells, is responsible for flagellin translocation into the host cytosol [20]. In the case of *Salmonella,* the T3SS encoded by the *Salmonella* Pathogenicity Island 1 (Spi1) is crucial for activation of caspase-1 and its downstream effects *in vitro*. In addition, the rod protein, PrgJ, which makes up the central core of the Spi1 T3SS also activates the inflammasome via murine Naip2 or human Naip1 and Nlrc4 [27] [28]. *Salmonella* is also capable of translocating flagellin via its Salmonella Pathogenicity Island 2 (Spi2) where it activates caspase-1 in an Nlrc4 dependent mechanism [29].

Caspase-1 dependent cell death, cytokine maturation and arachadonic acid metabolite production may all protect against *Salmonella* infection. Several studies indicate that caspase-1 substrates IL-1B and IL-18 contribute to host resistance against *Salmonella* infection *in vivo* in mice [30]; IL-1B may limit mucosal infection and IL-18 may limit systemic infection. Mice deficient in IL-1B and IL-18 had a higher bacterial burden in the spleen, MLN and Peyer's patches and decreased survival when infected orally with wildtype *Salmonella* [30]. The Nunez group also demonstrated that IL-1B is important for controlling *Salmonella in vivo*; mice deficient in IL-1B were more susceptible to orogastric infections [31]. However, the Aderem group demonstrated that caspase-1 controls infection by cell death and not through IL-1B and IL-18 production [32]. A recent publication by the Vance group showed an enigmatic phenotype dependent on flagellin and the Naip5/Nlrc4/Caspase-1

inflammasome, but independent on IL-1B and IL18. Activation of the inflammasome in this scenario leads to the release of prostaglandins and leukotrienes that are responsible for inflammation and vascular fluid loss [33]. The relevance of this novel downstream effect of the inflammasome for *Salmonella* infection is currently unknown.

ii) Extracellular flagellin recognition pathway

TLR5 activation by conserved regions of monomeric flagellin leads to NF-κB-dependent proinflammatory signaling in epithelial and immune cells [21](DC). As Salmonella breaks through the intestinal epithelial barrier, TLR5 receptors expressed on the basolateral surface of epithelial cells are believed to be a key component for recognizing invasive bacteria [34]. Once across the intestinal epithelial barrier, CD103+ DCs sense the presence of *Salmonella's* flagellin and secrete cytokines that stimulate IgA production and T helper cell differentiation [35]. The TLR5+ CD103+ DCs also activate innate lymphoid cells via production of IL-23, resulting in the production of IL-22 and other cytokines [36]. TLR5 stimulation has been associated with release of cytokines such as: i) CCL20, a strong DC chemoattractant, ii) IL-6 and TNF-α, inducers of fever, and acute liver response and chemoattractants iii) IL-8, neutrophil chemoattractant and iv) IL-10, immunosuppresor [37,38] [39]. In humans, epidemiological studies have associated loss of function alleles of TLR5 with increased susceptibility to pathogenic bacteria such as *Legionella pneumophila* [40]. In this study, we will explore the importance of TLR5 in *Salmonella* infection.

Flagellin/Flagellar apparatus

Bacterial flagellins are the building blocks that create the filament used for motility. The flagellar filament is composed of approximately 20,000 flagellin monomers, and more than 50 genes are involved in the regulation and functional expression of the flagellum [41]. A functional flagellum has been associated with virulence, systemic infection, persistence and inflammation in several pathogenic microbes including *S. typhimurium* and *Pseudomonas* [42-46]. The assembly of the flagellar apparatus and its filament is performed by complex transcriptional hierarchy. First, the environmental and global factors influence the expression of the flagellar master regulator, *flhDC* (Fig 1.4). The flhD and flhC proteins form a heteromultimeric complex that is a transcriptional activator of class II flagellar genes, most of which encode components of the basal body, hook and motor structures. Importantly, FliA, the class III gene sigma factor, and FlgM, the anti-sigma factor, are also made at this time, and FlgM binds FliA to block its activity. Once the basal body and hook are completed, FlgM is secreted and FliA is released to activate transcription of class III genes, which include the flagellin genes, leading to the fully functional flagellum. *flgM* deficient *Salmonella* cannot turn off flagellin production. We take advantage of the flgM mutant in our studies to examine the role of host innate flagellin recognition pathways during *Salmonella* infection.

Wildtype *Salmonella* downregulate flagellin *in vivo*, and we hypothesize that this helps *Salmonella* evade innate immune activation and the host immune system. Pathogenic bacteria rely on a number of strategies that allow them to evade immune surveillance by either modifying (LPS) or regulating the expression of molecules that could be targeted by the host. Perturbations in the fine regulation of flagellin and the flagellar apparatus have severe implications in the virulence of *Salmonella*. For example, deletion of *flgM* leads to attenuation of Salmonella in mice, characterized by increased lethal dose and long term survival [47-49]. We hypothesize that this phenotype is due to enhanced flagellin recognition by the innate immune system, culminating in attenuation of the bacteria and potentially clearance. Published data indicate that flagellin expression is repressed in systemic sites of infection [50,51], suggesting that *Salmonella* actively evades flagellin recognition pathways and immune surveillance. Expression of the flagellin gene, *fliC*, was found to be anatomically restricted. In the intestinal phase of the infection, flagellin production, and the motility it entails, could enhance colonization and colitis by *Salmonella* [52]. This finding was supported by *in vivo* studies using a GFP reporter driven by the *fliC* promoter in *Salmonella*, which showed no expression of GFP in the spleen and MLN, but high expression in the Peyer's patches in infected mice [53]. The presence of GFP in the Peyer's patches indicates that flagellin was being produced during the intestinal phase of infection, and the lack of GFP expression in the spleen demonstrated that flagellin expression was repressed in systemic organs. In addition, studies have shown that CD4+ T cells specific for

flagellin expand transiently during an acute *Salmonella* infection and accumulate in the Peyer's patches and MLN, but are absent in the spleen, even with a high bacterial burden [51]. The combined studies suggest that *Salmonella* expresses flagellin during the intestinal phase of infection where it can be detected by the immune system, and represses flagellin expression at systemic sites in order to evade immune detection. In this thesis we will capitalize on deletion of *flgM*, which allows us to cripple *Salmonella* so that it continues to express flagellin throughout the infection, in order to assess the importance of flagellin recognition *in vivo*.

Streptomycin pretreatment infection model

Streptomycin pretreatment is provides a model to study the intestinal phase of Salmonellosis in mice. Oral infection of mice with *Salmonella typhimurium* without the use of streptomycin is characterized by a limited intestinal phase of infection/colonization, which lacks a significant intestinal inflammatory response, and progresses rapidly to a systemic infection, resembling more closely *Salmonella typhi* infection in humans [54]. *Salmonella typhimurium* in mice is taken up by intestinal M cells and travels systemically to the MLN, spleen and liver using CD18+ macrophages [55,56]. The streptomycin pre-treatment model in mice generates a phenotype that resembles a *Salmonella typhimurium* infection in humans and cows, and is characterized by pronounced colitis [54]. Thus, the streptomycin pre-treatment model allows us to study *Salmonella*-induced mucosal inflammation in the mouse. An alternative model

that is also commonly used to study Salmonellosis and acute intestinal inflammation is the bovine ileal loop model which is neither cost-effective, nor easy to genetically manipulate [57-59]. For this reason we chose the mouse as our *in vivo* model system, since we could manipulate both host and pathogen genes to gain a better understanding of the role of innate immune detection of flagellin during *Salmonella* infection.

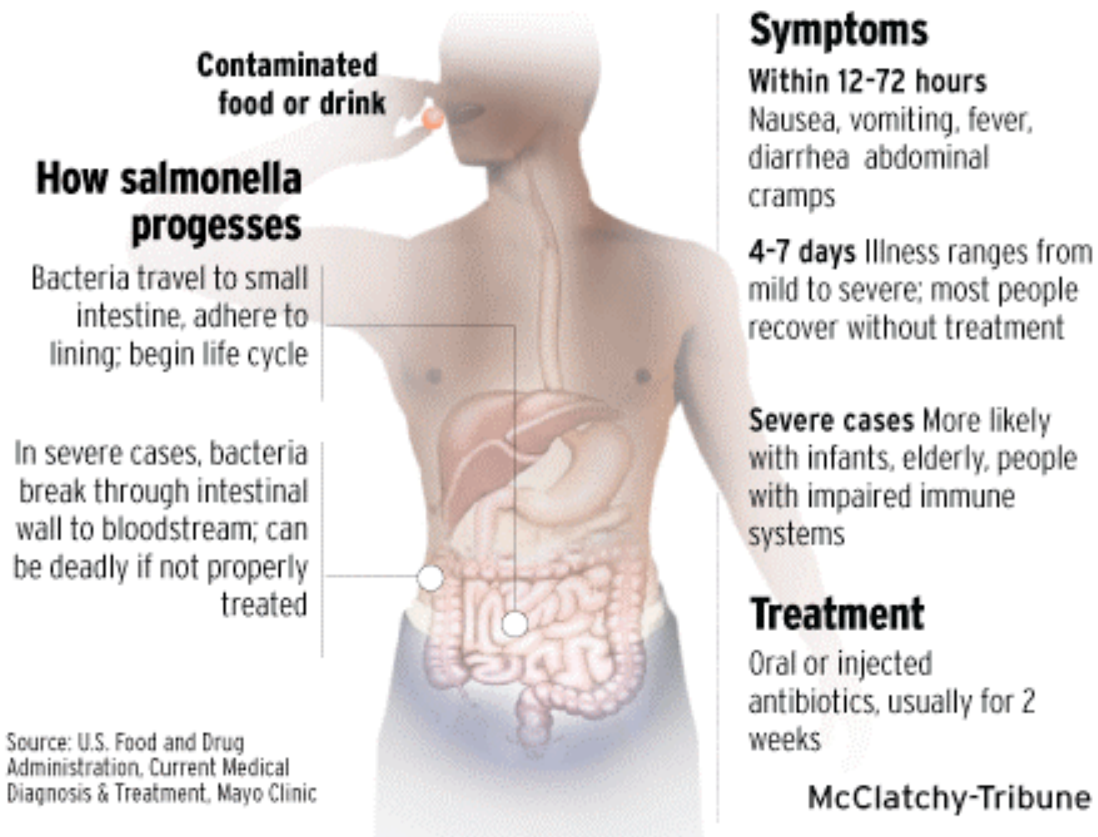

Figure 1.1: Salmonella infection in humans.
A schematic representation of Salmonella infection in humans, its progression, and common symptoms. The figure was produced by Mc Clatchy-Tribune with information from the U.S. Food and Drug Administration.

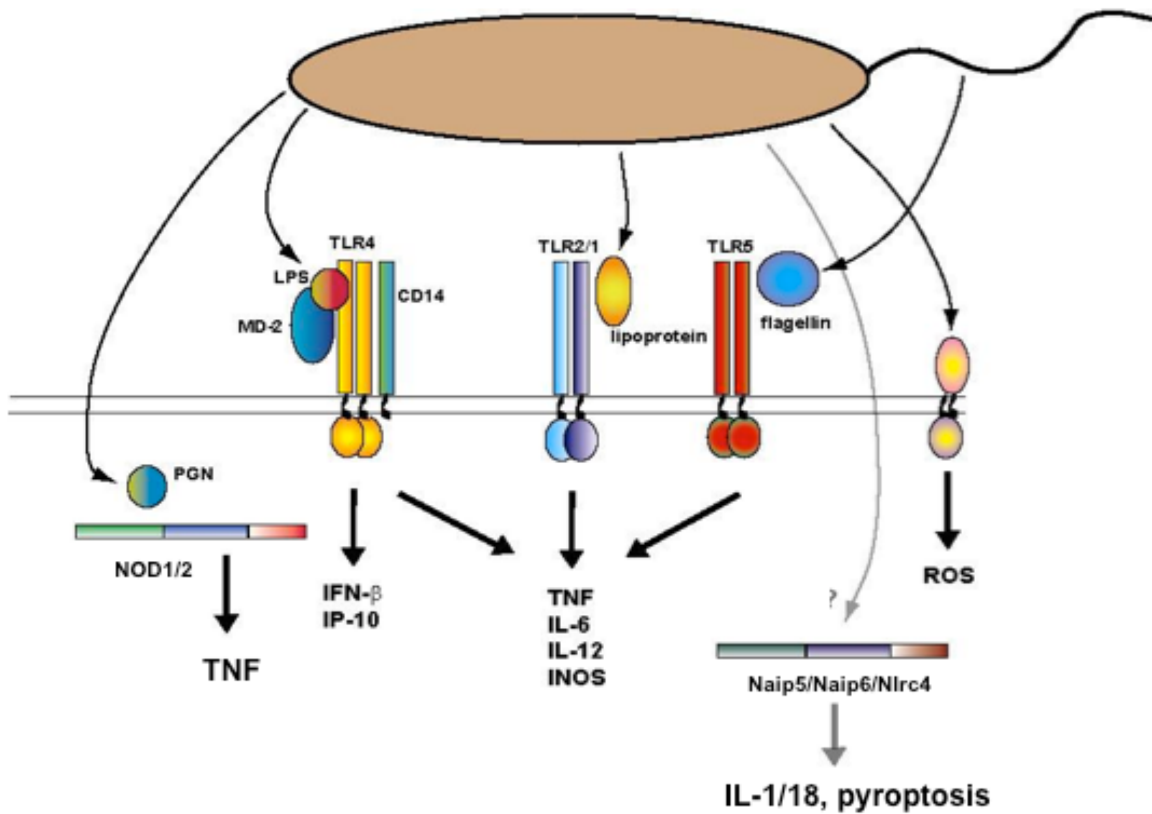

Figure 1.2: Innate immune recognition of Salmonella.
The innate immune system is capable of detecting several conserved motifs of Salmonella including bacterial wall components (i.e. LPS, peptidoglycan) using NOD1/2 and TLR 1/2/4, and flagellin by TLR5 and Naip5/Naip6/Nlc4/Casp1 dependent inflammasome.

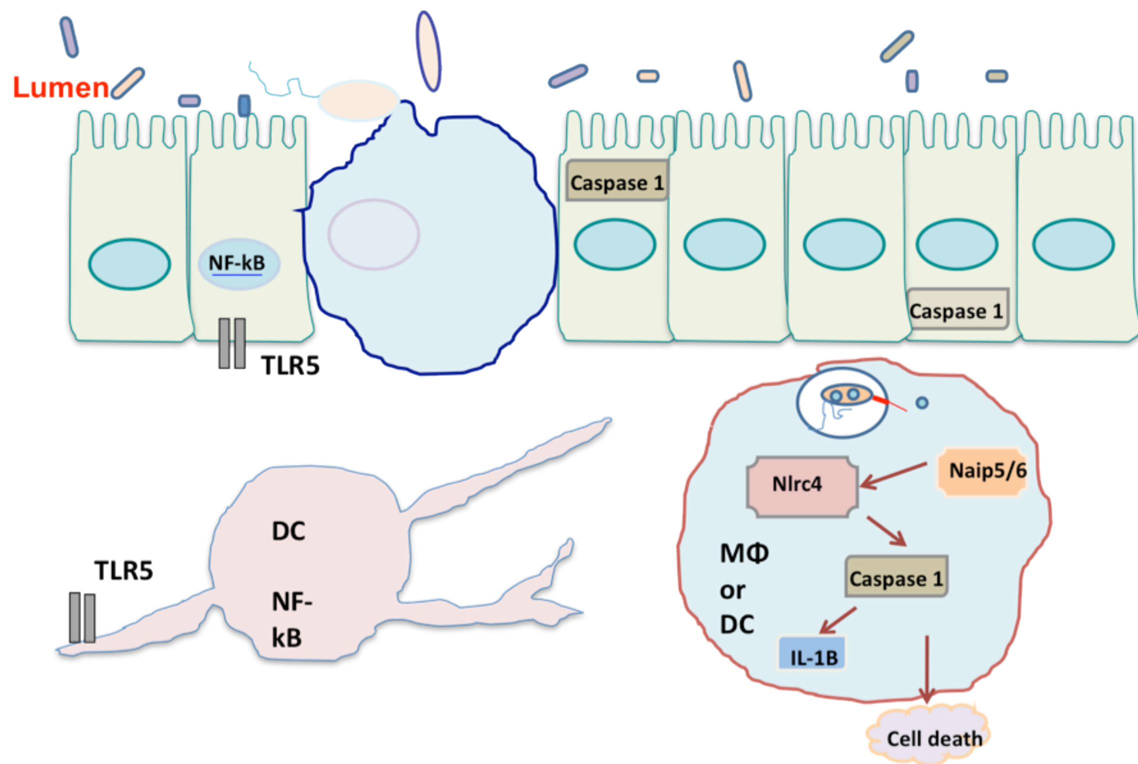

Figure 1.3: Innate immune recognition of Salmonella's flagellin in the gut.
Salmonella's flagellin is recognized in the gut by two independent flagellin recognition pathways. TLR5 present on a subset of intestinal dendritic cells and at the baso-lateral surface of intestinal epithelial cells sense flagellin and produce cytokines via NF-kB. Alternatively, Salmonella phagocytosed by macrophages or dendritic cells activate Naip5/Naip6/Nlrc4/Caspase1 by translocating flagellin to the cytosol of the host cell.

Figure 1.4: Production and regulation of the flagellar apparatus.
The production of the flagellar apparatus is under the regulation of flhDC operon. The heteromultimeric complex formed by flhDC leads sigma 70 factor dependent transcription of genes implicated in the formation of the hook-basal body (HBB) and flgM. flgM arrests the development of the flagellar apparatus until all necessary pieces are formed and properly assembled. At this stage, flgM is released to the extracellular environment and the flagellar apparatus is allowed to finalize the flagellar apparatus formation and the assembly of a flagellin rich filament.
The figure was produced by Fabienne F.V. Chevanceand Kelly T. Hughes. Nature Reviews Microbiology 2008.

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Chapter 2: Innate Immune Detection of Flagellin Positively and Negatively Regulates *Salmonella* Infection

Abstract

Salmonella enterica serovar Typhimurium is a flagellated bacterium and one of the leading causes of gastroenteritis in humans. Bacterial flagellin is required for motility and also a prime target of the innate immune system. Innate immune recognition of flagellin is mediated by at least two independent pathways, TLR5 and Naip5-Naip6/NlrC4/Caspase-1. The functional significance of each of the two independent flagellin recognition systems for host defense against wild type *Salmonella* infection is complex, and innate immune detection of flagellin contributes to both protection and susceptibility. We hypothesized that efficient modulation of flagellin expression *in vivo* permits *Salmonella* to evade innate immune detection and limit the functional role of flagellin-specific host innate defenses. To test this hypothesis, we used *Salmonella* deficient in the anti-sigma factor *flgM*, which overproduce flagella and are attenuated *in vivo*. In this study we demonstrate that flagellin recognition by the innate immune system is responsible for the attenuation of *flgM- S.* Typhimurium, and dissect the contribution of each flagellin recognition pathway to bacterial clearance and inflammation. We demonstrate that caspase-1 controls mucosal and systemic infection of *flgM- S.* Typhimurium, and also limits intestinal inflammation and injury. In contrast, TLR5 paradoxically promotes bacterial colonization in the cecum and systemic infection, but attenuates intestinal inflammation. Our results

indicate that *Salmonella* evasion of caspase-1 dependent flagellin recognition is critical for establishing infection and that evasion of TLR5 and caspase-1 dependent flagellin recognition helps *Salmonella* induce intestinal inflammation and establish a niche in the inflamed gut.

Introduction

Innate immune recognition of *Salmonella* is mediated by evolutionarily conserved receptors [pattern recognition receptors (PRRs)] that are capable of sensing conserved bacterial structures [pathogen associated molecular patterns (PAMPs)] that promote inflammatory and immune responses [18]. PRRs recognize a wide range of microbial ligands such as bacterial DNA, cell wall components (peptidoglycan and LPS) and flagellin. In this study, we focus on *Salmonella* flagellin recognition by the innate immune system. Bacterial flagellin has been studied for decades due to its importance in motility. Flagellin based motility and chemotaxis are important for the induction of acute colitis, and to compete with other microbiota for nutrients in the inflamed gut [52]; [60]. Flagellin is also a target of the innate immune system. There are at least two flagellin recognition pathways in mammals: i) cytosolic flagellin is detected by Naip5-Naip6/NlrC4/Caspase-1 and ii) extracellular flagellin is detected by Toll-like receptor 5 (TLR5) [19-21,27,34,61-63].

Mice detect a conserved site in the carboxy-terminus of flagellin using the intracellular receptors Naip5 and Naip6, leading to Naip5/6

association with Nlrc4 and the activation of caspase-1 [19-21,27,62]. Nlrc4 contains a caspase activating region domain (CARD) and can associate with caspase-1 in the absence of Asc, promoting caspase-1 autocleavage and cell death [20,64]. Association of the Nlrc4 inflammasome with Asc leads to efficient processing and secretion of IL-1B and IL-18 [29]. Caspase-1 protects mice against oral infection with *Salmonella*, which is dependent on IL-1B and IL-18 production and mediated by the Nlrc4 and Nlrp3 inflammasomes [29,30,65]. The Nlrc4 inflammasome also controls intraperitoneal infection of mice from *Salmonella* that overexpress flagellin, where Nlrc4-mediated protection is dependent on pyroptosis and independent of IL-1B and IL-18 [66]. The relevance of caspase-1 dependent flagellin detection during *Salmonella*-induced enterocolitis has not been fully elucidated.

TLR5 recognizes a conserved surface in the D1 domain of monomeric flagellin, and activates NF-κB dependent signaling in epithelial and immune cells [34,61,63,67,68]. TLR5 stimulation has been associated with release of inflammatory mediators, such as CCL20, a strong DC chemoattractant; IL-6 and IL-12/IL23p40; Reg3γ, an antimicrobial peptide; IL-8-like chemokines; and IL-10, an immunosuppresor [36-39]. Alexopoulo and colleagues found that TLR5 protected mice against *Salmonella* infection, but that this function was largely masked by TLR4 [69]. Akira and colleagues demonstrated that TLR5 recognition of flagellin promoted *Salmonella* infection and systemic spread [70]. Gewirtz and colleagues demonstrated that loss of TLR5 led to an increased basal inflammatory state and non-specific resistance to *Salmonella*

infection, suggesting that TLR5 interactions with the intestinal microbiota may promote *Salmonella* infection [71,72]. Thus the role of TLR5 in mucosal *Salmonella* infection is complex, and involves both homeostatic interactions with gut commensals that influence microbial composition and immune status, and detection of flagellated pathogens.

Flagellar assembly is under complex and tight regulation, with multiple signals that converge on the operon for the master regulator flhDC, as well as additional transcriptional, translational and post-translational mechanisms that control the expression of class II and III genes, and the assembly of the flagellum [41]. FlgM inhibits FliA, the sigma factor required for the transcription of class III flagellar genes [41]. Because flagellar assembly is costly, complex regulation may help conserve energy for growth in certain environmental conditions [73,74]. *Salmonella* selectively represses flagellin expression in systemic sites during infection, suggesting that active evasion of immune surveillance may also be critical for *Salmonella* to establish systemic infection [50-52]. During infection *Salmonella* has several mechanisms to sense the host environment and regulate gene expression. Some of these sensors, such as PhoPQ and ClpXP, down regulate flagellin synthesis [53,75,76]. Functional FlgM is also required to silence flagellin expression *in vivo*, and bacteria with deletions in *flgM* are attenuated during infection of mice in a flagellin-dependent manner [47]. We hypothesized that FlgM-dependent flagellin silencing is critical for *Salmonella* to evade innate immune

detection, and that either TLR5 or caspase-1 are responsible for the attenuation of *flgM⁻ Salmonella*.

Our studies demonstrate that TLR5 and caspase-1 have modest roles in host defense against wild type *Salmonella* infection, which is largely due to efficient FlgM-dependent silencing of flagellin expression. When flagellin production is dysregulated by deletion of *flgM*, TLR5 paradoxically limits cecal inflammation and promotes cecal colonization, but does not alter systemic dissemination. In contrast, *flgM- Salmonella* are efficiently cleared by the innate immune system in a caspase-1 dependent manner. In addition, caspase-1 is critical for limiting mucosal inflammation, and modulating the production of several cytokines and chemokines that may play cytoprotective roles in *Salmonella*-induced enterocolitis. Thus caspase-1 has ideal properties for innate immune defense at mucosal surfaces, and simultaneously limits bacterial dissemination and mucosal inflammation.

Methods:

Ethics Statement. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All protocols were approved by the Institutional
Animal Care and Use Committee of the University of Washington (protocol: 4031-01, Macrophage Biology).

*Bacterial strains.*The experiments were performed using wildtype *Salmonella* enterica serovar Typhimurium strain SL1344 (WT) (from Brad Cookson, University of Washington). The flagellin-deficient (*fliC*$^{-}$/*fljB*$^{-}$) strain in this same background was also a gift from Brad Cookson (University of Washington). A *flgM* deletion mutant was made in SL1344 using lambda-red technology [77]. The deletion was confirmed by PCR, and also transferred to the *fliC*$^{-}$/*fljB*$^{-}$ mutant using P22 phage [78]. The deletion of *flgM* was once again confirmed by PCR. A constitutive GFP expressing plasmid (pDW5: ptetA::gfp; *gfp* downstream of *tetA* promoter in pBR322); reference PMID: 16803592) was introduced in *Salmonella* for fluorescent microscopy studies, the plasmid was a gift from Brad Cookson (University of Washington). Bacteria were grown in Luria broth at 37°C with aeration.

Mouse infection. WT C57BL/6J (B6) mice were purchased from Jackson Labs and housed or bred in our facilities at the University of Washington. Caspase 1$^{-/-}$ [79] and Toll-like receptor 5 deficient mice (*TLR5*$^{-/-}$) [70] on a B6 background were bred in our specific pathogen free (SPF) animal facilities. Animals were housed individually or in groups of up to five animals under standard barrier conditions in individually ventilated cages. 8-14 weeks old mice were used for infections throughout this study. One day before infection, food was withdrawn 4 h before oral gavage with 20 mg of streptomycin in 0.1 ml of PBS [54]. Afterwards, animals were supplied food ad libitum. At 20 h after streptomycin treatment, food was withdrawn again for 4 h before the mice were orally infected with 1000 CFU *S*. Typhimurium

(delivered in 0.1 ml of PBS by gavage) or sterile PBS (control). The inoculum containing *Salmonella* was prepared by back-diluting an overnight culture 1:50 in LB + 50 µg/ml of streptomycin. After 4 hours, the concentration of bacteria was measured and diluted in cold PBS to a coentration of $1X10^4$ *CFU/ml*, and CFU of the inoculum was verified by plating on LB agar plates with 50 µg/ml streptomycin. Food was replaced immediately after gavage. Five days post-infection, mice were sacrificed by CO_2 asphyxiation, and blood and tissue (intestine, mesenteric lymph node, spleen, and liver) were promptly removed. Tissue samples from cecum and spleen were stored at -80 °C for RNA extraction. Additional samples from all tissues were fixed in 10% formaldehyde, or snap frozen in OCT medium for histopathological analysis. For some infections, cecal samples were also placed in Karnovsky' fixative for ultrastructural studies. Bacterial burden was assessed by weighing and homogenizing the tissues in PBS with 0.025% Triton X-100, and plating dilutions of the samples on MacConkey agar plates with streptomycin (50 ug/ml). For the homogenizing step, the ceca were scraped and blotted to remove fecal content.

Competitive Index infection. The procedure for competitive index experiments followed the mouse infection protocol described above with the following exceptions: 1) the inoculum prepared contained equal numbers (1000 CFU) of WT and *flgM-* Salmonella, the inoculum was verified by plating bacteria on antibiotic specific LB plates (flgM- Salmonella were kanamycin and streptomycin resistant (kan^r-str^r) and wildtype Salmonella were streptomycin resistant only

(kan^s-str^r); 2) dilutions of the homogenized tissues (liver, spleen, MLN, and cecum) were plated on both kanamycin/streptomycin (50 µg/ml each) and streptomycin (50 µg/ml) MacConkey agar plates; and 3) the competitive index (C.I.) of flgM-/WT *Salmonella* was determined by the following equation:

C.I. = CFU kan^r-str^r/ (CFU kan^s-str^r - CFU kan^r-str^r)

Quantitative histologic assessment of inflammation in cecum, small intestine, liver, spleen and MLN. The formalin-fixed tissue was embedded in paraffin using standard protocols. 4 µm thick sections were stained with hematoxylin and eosin using standard procedures. A blinded pathologist (KDS) examined the slides and scored them according to the following criteria. Scores were assigned for changes to the cecum as follows: **submucosal expansion (S)** - 0 = no significant change, 1 = <25% of the wall, 2 = 25-50% of the wall, 3 =>50% of the wall; **mucosal neutrophilic infiltrate (M)** - 0 = no significant infiltrate, 1 = mild neutrophilic inflammation, 2 = moderate neutrophilic inflammation, 3 = severe neutrophilic inflammation; **lymphoplasmacytosis (L)** - 0 = no significant infiltrate, 1= focal infiltrates (mild), 2= multifocal infiltrates (moderate), 3 = extensive infiltrates involving mucosa and submucosa (severe); **goblet cells (G)** - 0 = >28/HPF, 1 = 11-28/HPF, 2 = 1-10/HPF, 3 = <1/HPF; **epithelial integrity (E)** - 0 = no significant change, 1 = desquamation (notable shedding of epithelial cells into the lumen), 2 = erosion (loss of epithelium with retention of architecture), 3 = ulceration (destruction of lamina propria).

Western blot. Single colonies were grown overnight at 37 ^{o}C with constant shaking, back diluted the next day 1:50 and grown under similar conditions for 3 hrs. Equal numbers of cells were pelleted, the pellets and the supernatants were separated by SDS-PAGE, and transferred to Immobilon-P filters (Source, San Diego, CA). Filters were blocked with 5% non-fat milk, and Antibodies specific for *Salmonella* flagellin were used to detect the protein on the blot. The primary anti-FliC antibody was a monoclonal purified anti-FliC (Biolegend) at a concentration of 1:1000 diluted in PBS (Invitrogen) plus 1% BSA (SIGMA). The secondary antibody was a peroxidase-conjugated affinipure Goat anti-mouse IgG (H+L) (Jackson laboratories) at a concentration of 1:1000 diluted in PBS plus 1% BSA.

Motility assay. Motility was tested by using motility plates (LB with 0.3% agar). The radius of growth was measured at different time points during culture at 37 ^{o}C.

Macrophage cytotoxicity assay. Thioglycollate elicited peritoneal macrophages were plated in a 96-well plate at a concentration of 5 X 10^5 macrophages/well in RPMI 1640 medium with L-glutamine, 10% fetal bovine serum. *S.* Typhimurium was grown overnight in LB and backdiluted the next day 1:50 in LB medium and grown for 3-4 hrs. The bacteria was centrifuged (1000 rpm, 5 minutes) and the pellet resuspended to the final desired concentration of bacteria/ml. Macrophages were infected with the desired multiplicity of infection,

centrifuged at 250 x g for 5 minutes (to ensure infection of macrophages), and the infection was allowed to progress for an hour. Gentamicin (50 ug/ml) was added after an hour to kill extracellular bacteria. After an additional hour the supernatants were removed, and cytotoxicity was measured using Cytotox 96 kit (Promega).

Real-time PCR analysis of gene expression in cecum. Tissue was collected immediately post-euthanization and stored frozen at -80 ^{o}C. The tissue was later homogenized in Trizol reagent (Invitrogen, Carlsbad, CA), and total RNA was isolated according to manufacturer's procedure. Genomic DNA was removed with DNA-*free*™ (Ambion, Austin, TX), and cDNA was synthesized from 5 ug total RNA using Superscript III (Invitrogen, Carlsbad, CA). Multiplex real-time PCR was performed using primer probes sets for CCL20, CSCL1, CXCL2, IFNG, IL-12A, IL-12B, IL-17, IL-1B, IL-22, IL-23A, IL27, IL-6, LCN2, RG3G, TNF (all FAM labeled); the GAPDH control (VIC-labeled) was included in each reaction to normalize results. All reagents were from Applied Biosystems, and samples were analyzed on an ABI Prism 7900 using the fast protocol and reagents.

Measurements of serum cytokines by ELISA. Blood was collected from euthanized animals by cardiac puncture, and the serum was collected, centrifuged at 7000 g for 10 minutes and frozen at -20 ^{o}C. ELISAs for TNF-α, IL-12, IL-6 and IL-1B were performed on serum samples according to manufacturer's protocol (R&D systems, Minneapolis, MN).

Fluorescent staining. Frozen tissue blocks collected from mice infected with *Salmonella* containing a GFP plasmid were cut in 4 μm sections using a cryostat. Sections were fixed using acetone (-20 °C, Fisher Chemicals, Hanover Park, Il), washed with PBS (4 °C) and blocked with blocking buster™ (Innovex Bioscience, Redmond, CA) for 10 minutes at room temperature. The sections were washed with cold PBS and exposed to the F4/80 antibody (Rat anti-mouse, Serotec Inc, Raleigh, NC; diluted in PBS + 1% BSA 1:200) or TROMA-1 antibody (Rat anti-mouse , Developmental Studies Hybridoma Bank at the University of Iowa. Iowa city, IA; diluted in PBS + 1% BSA 1:200) for 2 hours at room temperature. The sections were then washed with cold PBS and an Alexa fluor 594 secondary antibody (donkey anti-rat, Invitrogen, Carlsbad, CA) was used at a concentration of 1:200 for 2 hrs. The sections were then washed with cold PBS and Vectashield™ mounting buffer with DAPI was added to cover all tissue (Vector labs, Burlingame, CA). *Salmonella* associated cells were quantified manually by counting the number of F4/80+ positive cells that co-localized with GFP-positive Salmonella; at least 100 cells were counted to determine the percentage of *Salmonella* carrying cells. The number of GFP-positive Salmonella associated with each F4/80+ cells was determined by counting the number of bacteria within the F4/80+ staining region for each infected cell (in WT cecum tissue, we counted the number of intracellular bacteria in 33 cells, in caspase-1 -/- cecum tissue, we counted 48 cells).

Electron Microscopy (EM). During dissection, the ceca of WT and caspase-1 -/- mice orally infected with *flgM-* was fixed in 1/2 strength

Karnovsky's, post fixed in osmium tetroxide and then embedded in Eponate-12 by standard EM procedures. Tissue processing for EM by the Pathology Research Service at the University of Washington, Seattle. For detailed instructions please refer to [80,81]

Lamina propria cell (LPC) isolation. Large bowel (cecum and colon) lamina propria cells were prepared as described previously (Mu reference). Large Intestines were cut open lengthwise and into 1cm pieces, then washed 2X with CMF solution [10 mM HEPES, 25 mM sodium bicarbonate (Sigma), and 2% FBS (Atlas Biological, Fort Collins, CO) in Ca2+, Mg2+-free HBSS (Invitrogen), pH 7.2]. Tissue was transferred into EDTA/DTT/FBS/CMF solution [1.3 mM EDTA (Invitrogen), 1mM DTT (VWR, Radnor, PA), 10% FBS, in CMF supplemented with 1X HGPG (5 mM HEPES, 2 mM L-glutamine (Fisher Scientific), 20 U/ml penicillin/20 ug/ml streptomycin (Fisher Scientific) and 20 ug/ml gentamicin (Invitrogen))]. After shaking at 250 rpm and 37°C for 20 min, samples were vortexed for 10s and detached epithelial cells and the supernatant were discarded. The tissue was washed twice with CMF and then strained using nylon mesh (70 mm nylon, Becton Dickinson (BD), San Jose, CA). LPC suspensions were prepared from this EDTA treated and de-epithelialized tissue by further incubation with collagenase solution [0.5 mg/ml of type II collagenase (Worthington, Lakewood, NJ), 1X HGPG and 5% FBS] at 37°C and shaking (250rpm) for 1 hr. At this time the tissue was completely digested, and the cell suspension was separated on a 40/80 Percoll gradient. After centrifugation for 20 min at 2,400 rpm at RT, the LPC were isolated from the interface of the

40/80 Percoll gradient. LPC were washed twice with RPMI 1640 medium supplemented with 100 U/ml penicillin/100 ug/ml streptomycin, 100 ug/ml gentamicin (Invitrogen), 10% FBS, and 50uM β-mercaptoethanol (Sigma). LP cells were counted using Trypan Blue exclusion to measure viability (≥95% viability) and approximately 1 x 10^5–10^6 cells were distributed into single wells of 96 well round bottom plates. For detection of intracellular cytokine production, LPC suspensions from flgM- *Salmonella* infected WT or Casp1-/- animals were cultured for approximately 4 hrs with 10 ug/ml Brefeldin A (Sigma) at 37°C prior to staining for flow cytometry.

Splenocyte isolation. Spleen cells were isolated by dispersing whole spleens in red blood cell lysis buffer using sterile glass slides. After 5 min incubation at RT, the cell suspension was diluted with 5 volumes of RPMI 1640 supplemented with 100 U/ml penicillin/100 ug/ml streptomycin, (Invitrogen), and 10% FBS. Splenocytes were counted using Trypan Blue exclusion to measure viability (≥95% viability) and approximately 1 x 10^5–10^6 cells were distributed into single wells of 96 well round bottom plates. For detection of intracellular cytokine production, splenocytes from flgM- *Salmonella* infected WT or Casp1-/- animals were cultured for approximately 4 hrs with 10 ug/ml Brefeldin A (Sigma) at 37°C prior to staining for flow cytometry.

Flow cytometry. Cells were treated with 2 mM EDTA in PBS for 10 min at 37 °C to detach adherent cells. Cells were washed with PBS + 1% BSA and then Fc receptors were blocked using α-CD16/32 for 15 min. For staining myeloid surface markers, cells were stained with α-

CD11c (FITC or PE-CY7), α-CDllb (PERCP-Cy5.5) α-I-A^b (FITC), Ly6G (APC), or α-CD103 APC. For intracellular staining, cells were washed with PBS with 0.02% azide, and resuspended in cytoperm/cytofix solution (BD) for 20 min on ice and then washed with Perm Wash (BD). Fixed and permeabilized cells were stained with α-TNF (Pacific Blue), or α-IL-12/23p40 (PE). For detection of Salmonella fixed and permeabilized cells were stained with α-Salmonella O antigen (Difco Laboratories, Detroit, MI), followed by donkey α-rabbit IgG (PE; Jackson Immunoresearch, West Grove, PA). Stained cells were washed several times with Perm Wash solution and resuspended in fresh 1% paraformaldehyde in PBS. We collected 1–$3x10^5$ gated events using a BD LSR II or Canto I flow cytometer. The data were analyzed using FlowJo software (Treestar, Inc., Ashland, OR). Unless mentioned, all antibodies used for flow cytometric analysis were purchased From BD Biosciences (San Jose, CA) or eBioscience (San Diego, CA).

Statistics. Significance was obtained by using the software GraphPad Prism (San Diego, CA). The Mann-Whitney test was used for all data where significance is shown. In all graphs, significance was established and represented using the following system: * = p<0.05, ** = p<0.01, *** = p<0.001. One way ANOVA was used when comparing three groups or more, using either the Dunn's (non-Guassian) or Bonferroni's (Guassian) multiple comparisons test. Statistical significance was represented as above.

Results

TLR5 promotes and Caspase-1 limits *Salmonella* infection in streptomycin-pretreated mice.

Flagellin recognition by the innate immune system is dependent on two pathways, the TLR5 pathway and a Naip5-6/Nlrc4/caspase-1 pathway. In order to establish the importance of each pathway, we infected *TLR5*$^{-/-}$ mice or *Casp1*$^{-/-}$ mice with WT *Salmonella*. Caspase-1 protects against systemic dissemination of WT *Salmonella* during oral infection in mice (Fig. 1 A-D). WT *Salmonella* colonized the systemic organs of *TLR5*$^{-/-}$ and WT C57BL/6 mice equally well, suggesting that WT *Salmonella* efficiently evade TLR5 recognition of flagellin (Fig. 1 E-H). Paradoxically, the cecal bacterial load was decreased in *TLR5*$^{-/-}$ mice, indicating a role for TLR5 in the regulating cecal colonization by *Salmonella* (Fig. 1 E).

Caspase-1 mediated protection is dependent on *Salmonella* flagellin. We next infected *Casp1-/-* mice with flagellin-deficient *Salmonella* to determine if flagellin is needed for enhanced virulence in *Casp1*$^{-/-}$ mice. Flagellin-deficient *Salmonella* colonized the cecum, spleen, liver and MLN of *Casp1*$^{-/-}$ and WT mice equally well, indicating that caspase-1 mediated control of *Salmonella* infection is dependent on flagellin expression by *Salmonella* (Fig. 2 A-D).

TLR5 promotes infection independent of Salmonella flagellin. To determine if flagellin is needed for the increased cecal colonization in WT relative to *TLR5*$^{-/-}$ mice, we infected these mice with flagellin-deficient bacteria. Flagellin-deficient *Salmonella* were even more

attenuated than WT bacteria in the *TLR5*$^{-/-}$ mice, and fewer bacteria were recovered from all organs (Fig. 2 E-H). Thus TLR5 promotes *Salmonella* infection independently of flagellin expression by *Salmonella*.

In vitro* characterization of *flgM*-deficient *Salmonella. O'Brien and colleagues determined that *flgM* is required for *Salmonella* virulence [47-49]. FlgM hypersecretion due to a mutation in *fliD* is also associated with decreased virulence [82]. The loss of virulence in FlgM defective *Salmonella* requires *fliA* and *fliC* [47], indicating that FlgM inhibition of FliA-dependent flagellin expression is necessary for virulence. To confirm these previous studies and extend these findings to *Salmonella* induced enterocolitis, we deleted the entire *flgM* gene from *S.* Typhimurium SL1344 by homologous recombination (see methods). The *flgM* deletion (referred to as *flgM-* throughout this paper) secreted more flagellin *in vitro* and had more cell-associated flagellin (Fig. 3 A), but did not affect growth *in vitro* (Fig. 3 B) or motility (Fig. 3 C). In addition*, flgM-* and wild type *Salmonella* killed macrophages equally well, and this was largely flagellin-dependent (Fig. 3 D). Finally, *flgM*- *Salmonella* produced more TLR5 stimulatory activity than WT *Salmonella*, consistent with greater flagellin production by this mutant (Fig. 3 E).

Deletion of *flgM* attenuates *S.* Typhimurium *in vivo*. Mice infected with *flgM-* had substantially and significantly less bacteria in their tissues, which was modest in the cecum (Fig. 4 A), and more pronounced in the spleen, MLN and the liver (Fig. 4 B-D). The ceca of

WT and *flgM-* infected mice were thick walled, pale and small (Fig. S1 A). Histologically, WT *Salmonella* infected mice exhibited prominent epithelial injury, edema, and leukocytic infiltration (Fig. 4 E). In contrast, the ceca of *flgM-* infected mice showed moderate inflammation, characterized by edema and moderate leukocyte infiltration, with good preservation of the epithelium (Fig 4 F). Quantification of cecal inflammation demonstrated that, *flgM- Salmonella* induced less inflammation relative to WT (Fig. 4 I). We measured gene expression in the cecal tissue from these mice. Compared to mock-infected animals both WT and *flgM*- infected mice demonstrated significantly increased expression of Cxcl1, Cxcl2, IL-1B, Reg3g, Lcn2 and TNF, and the *flgM*- infected mice also had increased levels of Ccl20, IFN-γ and IL-6 (Fig. S2 A). Mice infected with *flgM*- *Salmonella* had significantly increased Reg3g expression compared to WT infected mice (Fig. S2 A)

Systemically, *flgM-* infected mice had discrete foci of inflammation in the liver and spleen with no significant histologic evidence of hepatotoxicity (Fig. 4 H and data not shown), while WT infected mice exhibited severe hepatotoxicity, characterized by prominent vesicular change within the hepatocyte cytoplasm, and overall less inflammation (Fig. 4 G). *Salmonella* did not colocalize with hepatocytes suggesting that liver damage was indirect and not due to direct infection of hepatocytes (data not shown). Thus we hypothesized that the prominent hepatotoxicity was the result of a cytokine storm in the setting of systemic infection and sepsis [83]. Measurement of serum cytokines confirmed that WT infected mice

had higher levels of pro-inflammatory cytokines such as TNF-α and IL-6 (Fig. S3 A, S3 C respectively). Serum levels of IL-1B were similar in all infected groups (Fig. S3 B). In contrast IL-12/23p40 was higher in *flgM*$^{-}$ infected mice (Fig. S3 D).

The attenuated phenotype of *flgM-* is dependent on flagellin. To verify that the decreased bacterial burden and inflammation in *flgM-* infected mice was dependent on flagellin, we deleted the flagellin genes, *fljB* and *fliC*, in the *flgM-* bacteria. In the cecum the bacterial burden of *flgM*/flagellin-deficient *Salmonella* was comparable to that of *flgM-* (Fig. 5 A). The failure of the flgM/flagellin-deficient *Salmonella* to regain the modest growth advantage in the cecum may be due to loss of motility [52]. Deletion of the flagellin genes restored virulence to *flgM-* bacteria as evidenced by increased CFU in the MLN, liver and spleen (Fig. 5 B-D). The *flgM*/flagellin-deficient strain also induced hepatotoxic changes as seen with WT *Salmonella* (data not shown). Deletion of the flagellin genes restored inflammation and injury in the cecal mucosa, which was confirmed by histologic quantification, demonstrating decreased goblet cells and loss of epithelial integrity (Fig. 5 E and fig S1 B). Thus loss of *flgM* results in flagellin-dependent attenuation of *S.* Typhimurium, which is manifest as decreased mucosal inflammation, mucosal injury, hepatotoxicity and systemic dissemination of bacteria.

Caspase-1 is required for attenuation of *flgM*-deficient *Salmonella*. We next investigated the role of caspase-1 in flagellin-dependent attenuation of *flgM- Salmonella*. The bacterial burden in

the cecum of *Casp1*$^{-/-}$ mice was significantly increased compared to WT C57BL/6 mice (Fig. 6 A), and differences were even greater in the spleen, liver and MLN (Fig. 6 B, C, D respectively). Hepatotoxic changes were also observed in the liver of *Casp1*$^{-/-}$ mice, while WT mice exhibited only mild changes characterized predominantly by discrete foci inflammation (Fig. 6 G, H). Once again, the hepatotoxic changes correlated with higher serum levels of TNF (Fig. S4 A). We also observed higher serum levels of IL-1B and IL-6 and lower IL12/IL23p40 in *Casp1*$^{-/-}$ mice (Fig. S4 B-D). The ceca of all infected mice were thick walled, small and pale (Fig S1 C). However, compared to WT mice, *Casp1*$^{-/-}$ mice had more severe inflammation and injury in the cecum (Fig. 4 E, F), which even exceeded the histologic changes seen in WT mice infected with WT *Salmonella* (Fig 4 I) or *flgM*/flagellin-deficient *Salmonella* (Fig. 5 E). To further verify the importance of caspase-1 in the attenuation of *flgM*-deficient *Salmonella*, we performed competitive infection studies where mice were orally infected with equal numbers of WT and *flgM*-deficient *Salmonella*. The WT *Salmonella* outcompeted *flgM*-deficient *Salmonella* in the cecum, liver, spleen and MLN in WT mice, but not *Casp1*$^{-/-}$ mice (Fig. S5). These data suggest that caspase-1 dependent flagellin recognition is primarily responsible for the attenuated phenotype of *flgM- Salmonella*.

***Casp1*$^{-/-}$ mice have altered gene expression in the cecum**. *Casp1*$^{-/-}$ mice infected with *flgM- Salmonella* had higher bacterial counts in the cecum and higher histologic inflammatory scores compared to WT *Salmonella* infections in WT mice (Fig. 6 I). To assess the

importance of caspase-1 in mucosal immunity, we analyzed gene expression in ceca of WT and Casp1-/- mice infected with *flgM*-. Several genes that are involved in mucosal protection and immunity, such as Cxcl2 and IL-12A, retain levels of expression that are comparable to those observed in WT C57BL/6 mice and Lcn2, a gene that has been associated with gut inflammation [84], was detected at higher levels in *Casp1*$^{-/-}$ mice (Fig. S2 C). Paradoxically, *Casp1*$^{-/-}$ mice had significantly lower levels of Ccl20, Cxcl1, and Reg3γ (Fig. S2 C). This finding suggests that during *Salmonella*-induced enterocolitis caspase-1 selectively affects the expression of genes, of which some may be important for cytoprotection. Because Ccl20, Cxcl1 and Reg3γ are all known to be expressed by epithelial cells, and their expression is decreased in *flgM*-infected *Casp1*$^{-/-}$ mice but not *flgM*/flagellin- infected WT mice (Fig. S2 C), caspase-1 may also regulate cecal inflammatory responses in a flagellin-independent manner.

Caspase-1 limits intracellular growth of *Salmonella*. Using fluorescent microscopy we studied the location of *Salmonella* in the cecum and the contribution of caspase-1 *in vivo*. In the cecum, *Salmonella* does not reside in intestinal epithelial cells of WT mice as demonstrated by TROMA-1 staining and only rarely present in epithelial cells of *Casp1*$^{-/-}$ mice (Fig. 7 A, B respectively). This observation was confirmed by electron microscopy, where no bacteria were seen in epithelial cells of WT mice, and only few cytoplasmic bacteria were detected in the epithelial cells of *Casp1*$^{-/-}$ mice (Fig. S6). In addition these studies confirmed better

preservation of the epithelium in WT compared to *Casp1*$^{-/-}$ mice (Fig. S6). *Salmonella* colocalized predominantly with TROMA-1 negative cells in the mucosa and submucosa of WT and *Casp1*$^{-/-}$ mice. Most *Salmonella* colocalized with F4/80+ cells indicating that phagocytes were the main host cells for *Salmonella in vivo* (Fig 7 C, D and Fig. S7 A, B). Quantification of *Salmonella* associated with F4/80+ cells revealed that *Casp1*$^{-/-}$ mice had significantly more infected cells and more bacteria per cell (Fig. S7 C). Electron microscopy studies confirmed this observation, and revealed that most of the bacteria in the lamina propria and submucosa of WT mice were extracellular and adjacent to cell debris, with only rare intracellular bacteria present within simple vesicles (Fig. S8). In contrast, the *Casp1*$^{-/-}$ lamina propria cells often harbored numerous bacteria that were frequently associated with complex and heterogeneous vacuoles, and occasionally free in the cytoplasm (Fig. S7 D and Fig. S9). These data suggest that caspase-1 controls intracellular bacterial growth *in vivo*. Immunofluorescence microscopy analysis of the MLN, spleen and liver revealed that *flgM*- *Salmonella* were also present in F4/80+ cells in *Casp1*$^{-/-}$ mice; bacteria were not detected in these organs of WT mice (data not shown).

Caspase-1 regulates the composition of immune cells within tissues and intracellular *Salmonella* growth. Lamina propria cells were recovered from flgM- infected *Casp1-/-* and WT mice, and analyzed by flow cytometry. CD11b and CD11c staining revealed

that these mice had similar proportions of cells, but phenotypic differences existed in the CD11c^{+}/CD11b^{lo} DC populations; *Casp1-/-* mice retained the CD103+ DC population and this population was depleted in the WT mice (Fig. S10). Within the lamina propria, the CD11c^{+}/CD11b^{lo} cells were the primary producers of IL-12/23p40, and the lymphoid population was the primary producer of TNF for both WT and *Casp1-/-* mice (Fig. S11). The spleen of *Casp1-/-* mice showed marked increases in the CD11c^{-}/CD11b^{hi} population, which was composed predominantly of Ly6C^{hi}, I-Ab^{+} cells in the *Casp1-/-* mice, consistent with inflammatory macrophages; in WT spleens the CD11c^{-}/CD11b^{hi} population was predominantly Ly6C^{int}, I-Ab^{-}, consistent with neutrophils (Fig. S12 and S13). The CD11b^{hi} and CD11b^{int} cells were the primary producers of TNF, and more of these cells produced TNF in the *Casp1-/-* mice compared to WT mice (Fig. S12 C - F). This same population contained *Salmonella*, which was detected in substantial proportions of these cells in the *Casp1-/-* mice (Fig. S13). As in the lamina propria, the CD11c^{+}/CD11b^{lo} cells were the major producers of IL-12/23p40 (Fig. S12 G, H).

The innate immune receptor TLR5 plays a complex role in the attenuation of *flgM^{-} Salmonella*. We hypothesized that innate immune detection of flagellin by TLR5 may also contribute to the attenuated phenotype of *flgM- S. typhimurium*. We first tested the role of TLR5 by infecting WT and *TLR5$^{-/-}$* mice with *flgM- Salmonella*. Similar to WT *Salmonella* infection (Fig.1 E, F, G, H), bacterial burden in the spleen, liver and MLN was indistinguishable between WT and

TLR5$^{-/-}$ mice (Fig. 8 A, B, C, D), indicating that the attenuation mediated by flgM deficiency is not due to flagellin detection by TLR5. Similar to WT *Salmonella* infection (Fig. 1 E), *TLR5$^{-/-}$* mice also had lower bacterial burden in the cecum compared to WT mice (Fig 8 A), consistent with our previous finding that TLR5 promotes cecal colonization of *Salmonella*. The ceca of all mice were inflamed, small and pale (Fig. S1 D). Histologically, the cecum (Fig. 8 E, F) and the liver (Fig. 8 G, H) of WT and *TLR5$^{-/-}$* mice were similar, although histologic quantification showed a modest but significant increase in cecal inflammation in *TLR5$^{-/-}$* mice (Fig. 8 E). Analysis of gene expression in the cecum of WT and *TLR5$^{-/-}$* mice showed no significant differences in all genes tested (Fig. S2 D). Serum cytokines in WT and *TLR5$^{-/-}$* showed similar levels of TNF-α, IL-1B, IL-12/23p40 in both groups; IL-6 was increased in *TLR5$^{-/-}$* mice (Fig. S14). To confirm that TLR5 promotes cecal colonization of flgM- *Salmonella* independent of *Salmonella*'s flagellin; we infected WT and *TLR5$^{-/-}$* mice with *flgM*-/flagellin- *Salmonella*. Once again, these flagellin-deficient bacteria were even more attenuated in the *TLR5$^{-/-}$* mice (Fig. S15), providing further evidence that the host protection provided by TLR5-deficiency is independent of flagellin expression by the pathogen, *S*. Typhimurium.

Discussion

In this study we demonstrate the importance of flagellin regulation by *Salmonella* and define the role of host flagellin recognition pathways in controlling systemic dissemination of bacteria, inflammation and

tissue injury. Proper flagellin regulation is paramount for the infectious life cycle of *Salmonella*. *Salmonella* downregulates flagellin production in systemic sites in mice [50,51] and disruption of flagellin regulation reduces bacterial burden and increases survival of mice [47]. Previous studies have demonstrated that deletion of *flgM*, the anti-sigma factor, leads to overproduction of flagellin and flagella *in vitro*, and attenuates *Salmonella in vivo* [47]. Our studies demonstrate that attenuation of flgM-deficient *Salmonella in vivo* is due to recognition of flagellin by the innate immune system, and predominantly by caspase-1 dependent pathways.

The role of TLR5 during *Salmonella* infection is controversial. TLR5 deficiency in mice has been associated with both increased and decreased susceptibility to WT *Salmonella* infections [69,70,72]. Our studies indicate that *TLR5*$^{-/-}$ mice have a complex phenotype. Similar to the findings of the Gewirtz's and Akira's groups [70,71], we found that *TLR5*$^{-/-}$ mice have lower CFU in the MLN during WT *Salmonella* infection. Akira and others have demonstrated that TLR5 plays an important role in *Salmonella* trafficking by a subpopulation of dendritic cells from the cecum to the MLN, thus providing a potential explanation to our observations. More intriguingly, flagellin^{-} *Salmonella* were even more attenuated in *TLR5*$^{-/-}$ mice; Vijay-Kumar et al. also reported increased survival of *TLR5*$^{-/-}$ mice infected with aflagellate *Salmonella* compared to WT mice [71]**.** These data suggest that recognition of *Salmonella's* flagellin by TLR5 is not required for enhanced virulence of *Salmonella* in WT mice compared to *TLR5*$^{-/-}$ mice. Vijay-Kumar et al. attributed the enhanced resistance

of *TLR5*$^{-/-}$ mice to an increased basal inflammatory state of *TLR5*$^{-/-}$ mice. Consistent with this hypothesis, we found that infected *TLR5*$^{-/-}$ mice had increased histologic evidence of inflammation in the ceca. However, our gene expression data showed no significant differences between infected TLR5 sufficient and deficient mice and serum cytokine measurements revealed only a modest increase in IL-6 in the TLR5-/- mice (Fig. S2 D, Fig. S14 C). In addition, we have never observed spontaneous colitis in our *TLR5*$^{-/-}$ mouse colony. Thus similar to Vijay-Kumar et al, we find that TLR5-deficiency protects mice against *Salmonella* infection, and that this protection does not require flagellin expression by *Salmonella*. *TLR5*$^{-/-}$ mice have recently been demonstrated to have an altered gut microbiota that promotes metabolic syndrome [72], and it is possible that an altered microflora or altered interactions between the host and the gut microbiota in *TLR5*$^{-/-}$ mice could establish a more effective barrier against *Salmonella* infection. Thus *Salmonella may be* subverting a TLR5-dependent host response in the gut to gain a competitive advantage over the microflora [54,85]. Further studies are required to elucidate how TLR5 influences *Salmonella* infection in the absence of flagellin expression by *Salmonella*.

Caspase-1 protected mice against WT *Salmonella* infection (Fig. 1), consistent with previous reports [30,65]. The deletion of flgM substantially magnified the protective capacity of caspase-1 as evidenced by 100-10,000 fold greater tissue CFU in *flgM- Salmonella* infected Casp1-/- mice compared to WT mice (Fig. 6). Our results suggest that flagellin recognition by the inflammasome during WT

Salmonella infection limits *Salmonella* spread to distant sites, and that *flgM*-dependent evasion of flagellin recognition by the inflammasome is necessary for *Salmonella* virulence. We compiled all of the CFU data from experiments that we performed using WT, TLR5-/- and Casp1-/- mice in infections with WT, flagellin-null and *flgM*- *Salmonella* (Fig. S16) This analysis reveals that dysregulated flagellin production profoundly attenuates *Salmonella* infection in all tissues in a caspase-1 dependent manner. In the absence of caspase-1, the *flgM* deletion has essentially no phenotype, and behaves nearly identical to wt *Salmonella*. Intriguingly, both wt and *flgM*- *Salmonella* are more virulent than flagellin- *Salmonella* in caspase-1 mice. The added virulence property that flagellin bestows upon *Salmonella* in the absence of caspase-1 may be motility, or possibly some other functional interaction between flagellin and the host. The most parsimonious explanation for modest increase in virulence of flagellin- *Salmonella* relative to WT Salmonella in WT mice is that flagellin- *Salmonella* are evading inflammasome detection at the cost of losing a flagellin-dependent virulence property, such as motility. Inflammasome detection of flagellin has only been convincingly demonstrated for rodents. Because inflammasomes in humans and many other animals are incapable of flagellin detection [27], some host-restricted *Salmonella* strains, such as *S. typhi*, are not under the same selective pressures to repress flagellin expression during infection.

The composite data also illustrate that TLR5 does not contribute to the attenuation of *flgM*- *Salmonella*, and that loss of TLR5 leads to

paradoxical protection against *Salmonella* infection, which is most evident in the cecum.   These experiments illuminate the complexity of host-pathogen interactions, and how the complex interplay between the host, pathogen and microbiota influence the outcome of infection. A more precise understanding of host-pathogen interactions will need to take into account the microbiota, and control for differences in microbiota that influence host-pathogen interactions.

Recently, murine TLR11 has been proposed as another flagellin receptor, and has been shown to protect mice against *Salmonella* infection and restrict *S. typhi* from growth in mice [27,86,]. Interestingly, the virulence defect *flgM*- *Salmonella* is completely complemented by caspase-1 deficiency, suggesting that TLR11 does not contribute to the attenuation of flgM- *Salmonella*. Additional studies will be needed to clarify the role of TLR11 in this model system.

FlgM- Salmonella infections in mice demonstrate the importance of proper flagellin regulation for virulence, systemic dissemination and inflammation. Cecal inflammation was diminished in *flgM-* infections compared to WT indicating a role for flagellin recognition in regulating inflammation and injury at mucosal surfaces. It is possible that excess stimulation of TLR5 or the inflammasome during mucosal infection leads to the preservation of mucosal integrity, which is further enhanced by the better control of the bacterial infection. *Casp1*$^{-/-}$ mice had more severe inflammation and injury in the cecum and higher bacterial burden in all organs indicative of caspase-1 role

in protecting mucosal surfaces and containing infection. Surprisingly, *Casp1*$^{-/-}$ mice had a selective decrease in pro-inflammatory gene expression in the cecum (Fig. S2 C), suggesting that caspase-1 also promotes inflammatory responses in the cecum that may be important for limiting tissue injury. Caspase-1, as well as Nlrc6 and Asc, limit mucosal injury in dextran sodium sulfate induced colitis through the modulation of the intestinal microbiota [27]. Our data indicate that caspase-1 also limits mucosal injury in *Salmonella* infection, and this may be mediated through caspase-1-dependent control of *Salmonella* growth, through caspase-1 regulation of mucosal inflammatory responses, or a combination of these factors. Further studies will be needed to determine which inflammasomes protect the gut during *Salmonella* infection, whether alterations in the gut microbiota contribute to inflammation and injury, and whether Asc-dependent IL-1 and IL-18 are required to protect against mucosal *Salmonella* infection and preserve epithelial integrity.

Using a heterologous Spi2 promoter to drive the expression of flagellin and FliS, the flagellin chaperone, Ed Miao and colleagues also observed a strong role for caspase-1 in sensing flagellin during intraperitoneal infections [66]. In their study, neither TLR5 nor IL-1/IL-18 played a significant role in limiting *Salmonella* growth, and attenuation of their *Salmonella* strain was dependent on Nlrc4/caspase-1 and pyroptosis [66]. It is likely that pyroptosis also contributed to attenuation of flgM-deficient *Salmonella* infection, and this is supported by the electron microscopy studies demonstrating extracellular *Salmonella* adjacent to dead cells and cellular debris

within the tissue of WT mice. In addition, our flow cytometry studies show a loss of CD103+ dendritic cell populations during *flgM-Salmonella* infection in WT mice relative to *Casp1*$^{-/-}$ mice. This suggests that lamina propria CD103+ DC, which have been demonstrated to be the first cells infected by *Salmonella* in the Peyer's patches [87], may be eliminated by pyroptosis in WT mice, and that the elimination of this cellular niche may be important for clearance of *Salmonella* by other leukocytes. Evasion of pyroptosis may provide a safe-haven for *Salmonella* to grow within phagocytes, as seen in the *Casp1*$^{-/-}$ mice. Systemically, the evasion of inflammasome permits intracellular bacterial growth and results in excessive stimulation of the immune system, as reflected by the massive accumulation of TNF-producing inflammatory macrophages in the spleen of *Casp1*$^{-/-}$ mice (Fig. S12 C - F).

It has been recently demonstrated that the *Casp1*$^{-/-}$ mice used in this study are also deficient in Casp11 (also referred to as Casp4), and that Casp11 deficiency in the context of Casp1 deficiency is protective against *Salmonella* infection. The model predicts that Casp11 induced cell death in the absence of Casp1-dependent IL-1B and IL-18 production increases host susceptibility to *Salmonella* infection [88]. This is thought to be manifest by Casp11-mediated killing of phagocytes and release of *Salmonella*, without IL-1B mediated recruitment of neutrophils to limit *Salmonella* infection. We noted that many of the intracellular *Salmonella* seen in *Casp1*$^{-/-}$ (and *Casp11*$^{-/-}$) mice were associated with complex phagosomes, suggesting that autophagy/xenophagy may be contributing to host

defense in the combined absence of Casp1 and Casp11. Further studies will be needed to determine the interaction between these caspases and the autophagy pathway in the control of *Salmonella* infection.

Altogether, our data indicate that regulation of flagellin production by *Salmonella* is critical to evade innate immune detection, and in particular caspase-1 dependent responses. Caspase-1 is critical for controlling systemic dissemination, severe inflammation and the integrity of the mucosal barrier.

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Wildtype Salmonella infection:

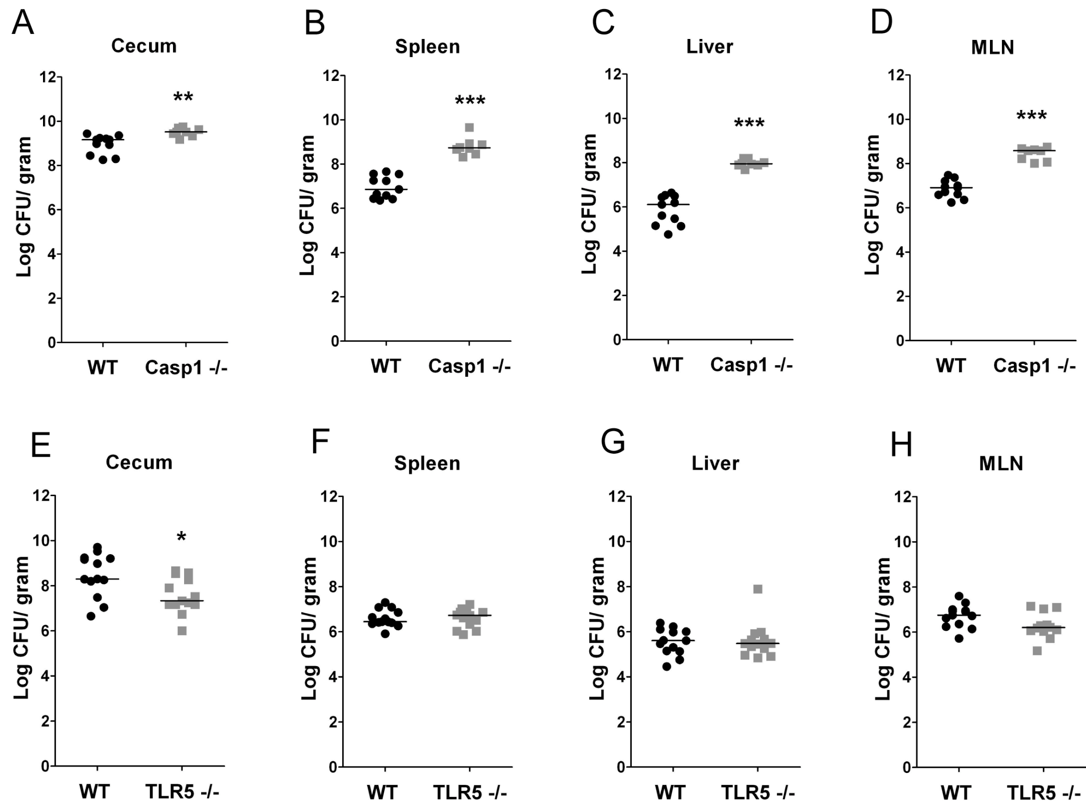


Figure 1. Wildtype *Salmonella* efficiently evades flagellin detection during acute mucosal infection in streptomycin-pretreated mice.
Bacterial burden of WT C57BL/6 (n=11) and caspase-1 -/- (n=8) mice infected with 1000 cfu WT SL1344 *Salmonella* in the cecum (A), spleen (B), liver (C), MLN (D). Bacterial burden of WT C57BL/6 (n=12) and TLR5 -/- (n=13) mice infected with 1000 cfu wildtype *Salmonella* in the cecum (E), spleen (F), liver (G), MLN (H). Figures A-D are the combined data from two independent experiments. Figures E-H are the combined data from three independent experiments. Mann-Whitney test. * = p<0.05. ** = p<0.01. *** = p<0.001.

Flagellin deficient Salmonella infection:

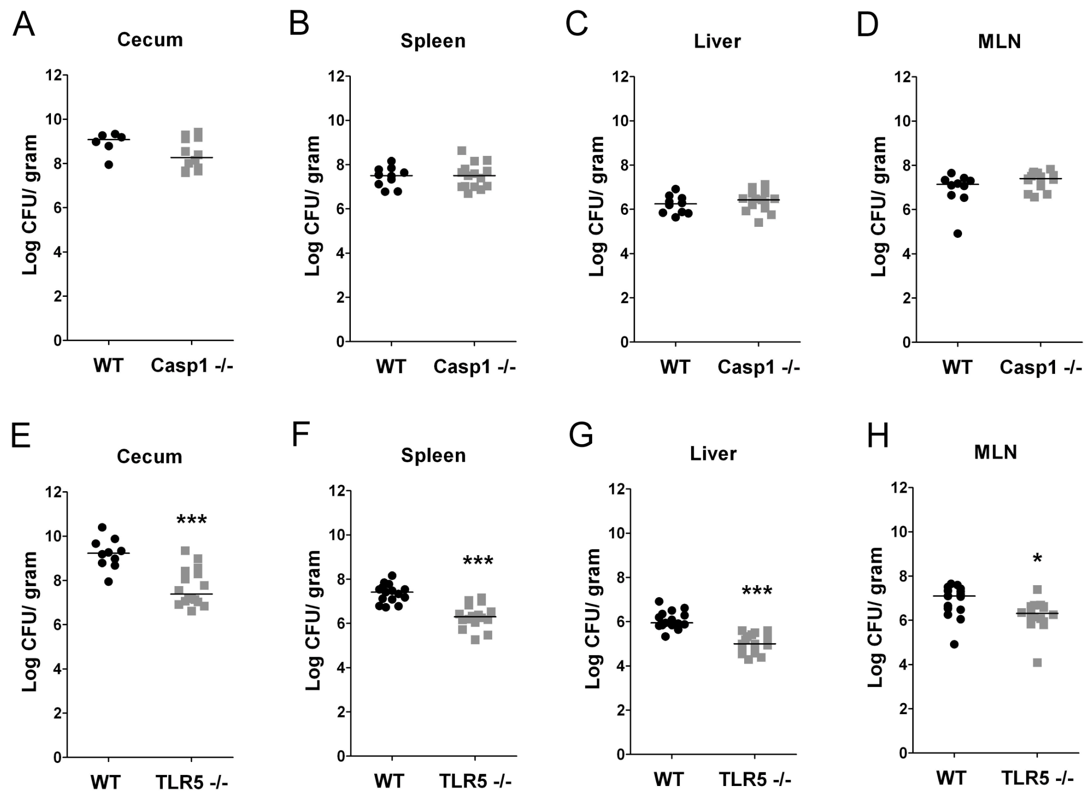


Figure 2. Flagellin detection accounts for the caspase-1 increased susceptibility during acute mucosal infection in streptomycin-pretreated mice. Bacterial burden of WT C57BL/6 (n=10) and caspase-1 -/- (n=16) mice infected with 1000 cfu flagellin^{-} *Salmonella* in the cecum (A), spleen (B), liver (C), MLN (D). Bacterial burden WT C57BL/6 (n=15) and TLR5 -/- (n=16) mice infected with 1000 cfu flagellin^{-} *Salmonella* in the cecum (E), spleen (F), liver (G), MLN (H). Figures A-D are the combined data from two independent experiments. Figures E-H are the combined data from three independent experiments. Mann-Whitney test * = p<0.05. *** = p<0.001.

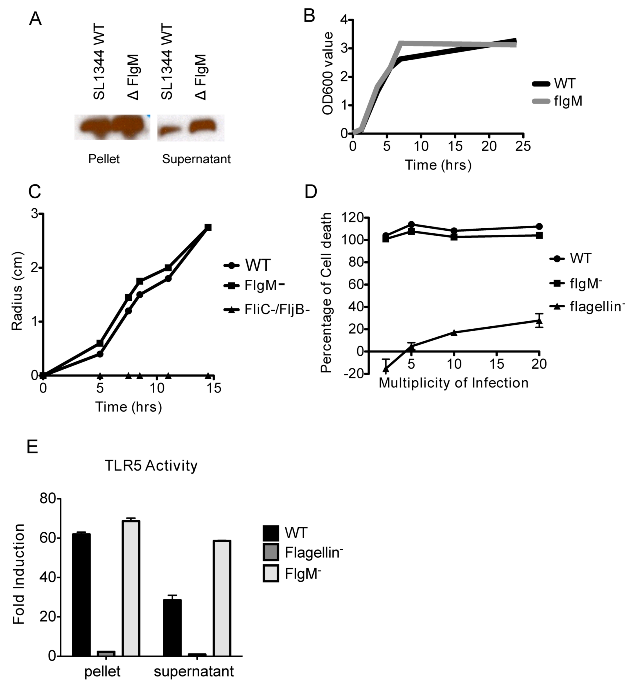
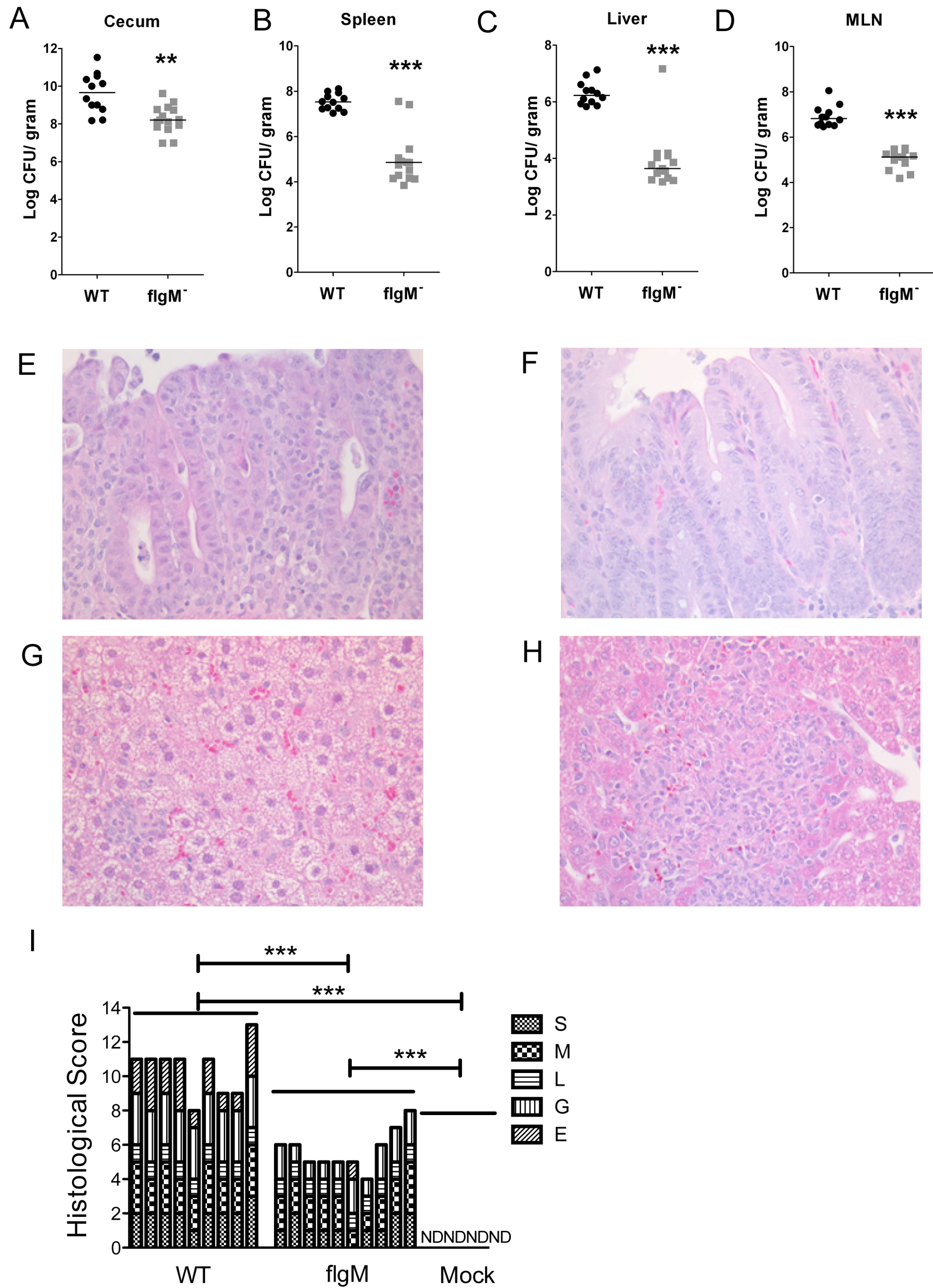


Figure 3. *FlgM$^-$ Salmonella* overproduce flagellin *in vitro*. (A) Western blot for flagellin from WT and *flgM$^-$ S.* Typhimuirum cell pellets or supernatant; 1.5 X 10^8 cell equivalents were loaded in each lane. (B) Motility of *Salmonella* measured by using a 3 g/L of agar LB plate grown at 37 C. (C) Bacterial growth in LB broth measured by OD 600. (D) *Salmonella* induced cell death in thioglycollate elicited peritoneal macrophages measured by LDH release assay. (E) TLR5 activity measured using an NF-κB luciferase reporter CHO cell assay; MTLR5-CHO cells were stimulated with 1 X 10^5 heat killed cells (pellet), or 1 X 10^5 cell equivalents supernatant from WT or flgM- bacteria. Data are representative of two to three independent experiments.


A
Cecum
Log CFU/ gram
WT
flgM-
**
B
Spleen
***
C
Liver
***
D
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***
E
F
G
H
I
Histological Score
S
M
L
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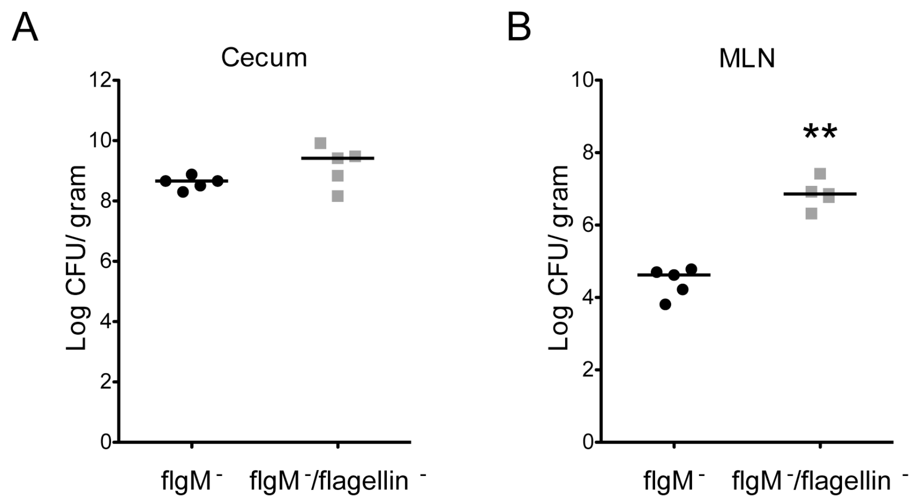
NDNDNDND
WT
flgM
Mock

Figure 4. *flgM⁻ Salmonella* has an attenuated *in vivo* phenotype. C57BL/6 mice were infected with 1000 CFU of either WT SL1344 (n=12) or *flgM⁻* (n=15) *Salmonella*. (A) Bacterial burden in the cecum. (B) Spleen. (C) Liver. (D) MLN. Representative histology of the cecum (E+F) and liver (G+H) infected with WT (E+G) or *flgM⁻ Salmonella* (F+H). (I) Histological scores for changes in the cecum. Submucosal expansion (S); mucosal neutrophilic infiltrate (M); lymphoplasmacytosis (L); goblet cells (G); epithelial integrity (E). Liver images (400X magnification), cecal images (200X magnification). Figures A-D are the combined data from three independent experiments. Mann-Whitney test. ** = p<0.01, ***= p<0.001


A
Cecum
Log CFU/ gram
12
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8
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4
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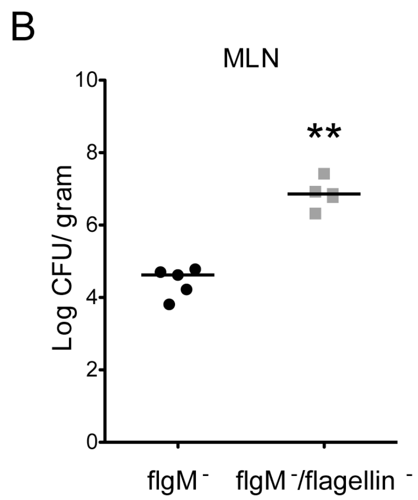
flgM -
flgM -/flagellin -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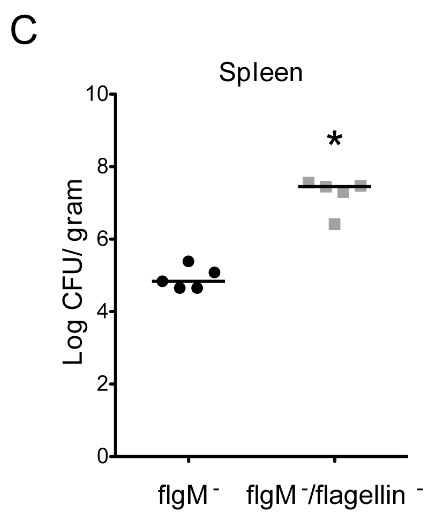
C
Spleen
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Log CFU/ gram
10
8
6
4
2
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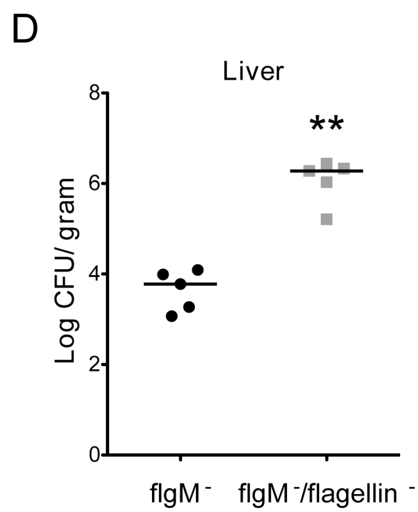
flgM -
flgM -/flagellin -


D
Liver
**
Log CFU/ gram
8
6
4
2
0
flgM -
flgM -/flagellin -

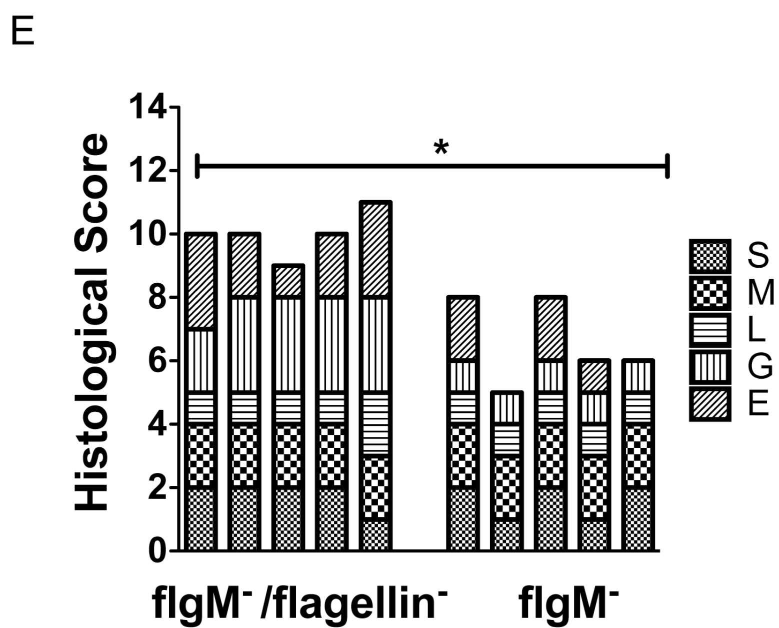
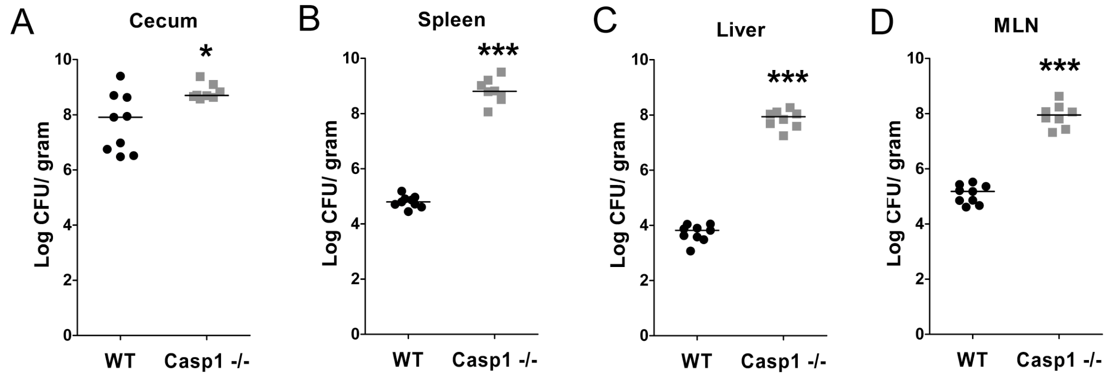
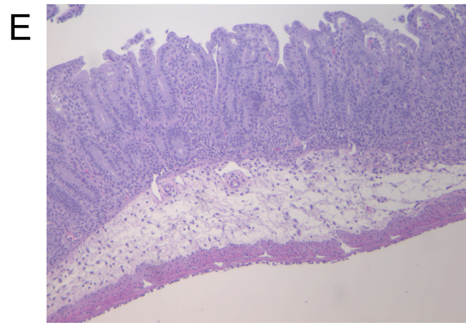
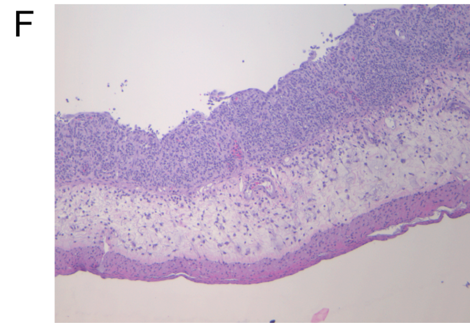

E
Histological Score
14
12
10
8
6
4
2
0
*
S
M
L
G
E
flgM- /flagellin-
flgM-

Figure 5. *flgM⁻ Salmonella* attenuated phenotype is dependent on flagellin. C57BL/6 WT mice were infected with 1000 CFU of either *flgM⁻/flagellin⁻* (n=5) or *flgM⁻* (n=5) *Salmonella*. Bacterial burden in the cecum (A). MLN (B). Spleen (C). Liver (D). (E) Histological scores for changes in the cecum in cecal pathology as described in Fig. 4. Figures A-D represent data from one experiment (*flgM⁻* n= 5; *flgM⁻/flagellin⁻* n=5). Mann Whitney test. * = $p<0.05$, **= $p<0.01$, *** = $p<0.001$.


A
Cecum
*
Log CFU/ gram
10
8
6
4
2
0
WT
Casp1 -/-
B
Spleen
***
Log CFU/ gram
WT
Casp1 -/-
C
Liver
***
Log CFU/ gram
WT
Casp1 -/-
D
MLN
***
Log CFU/ gram
WT
Casp1 -/-


E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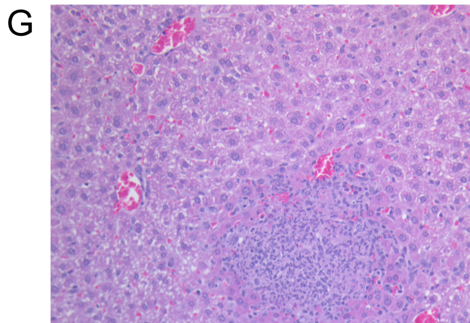
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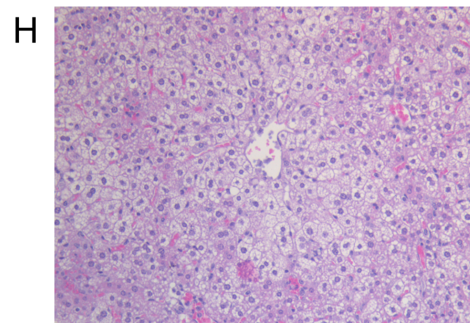
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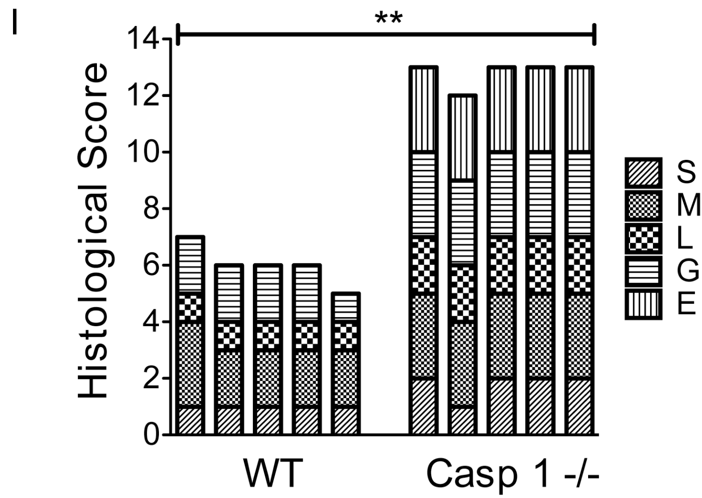
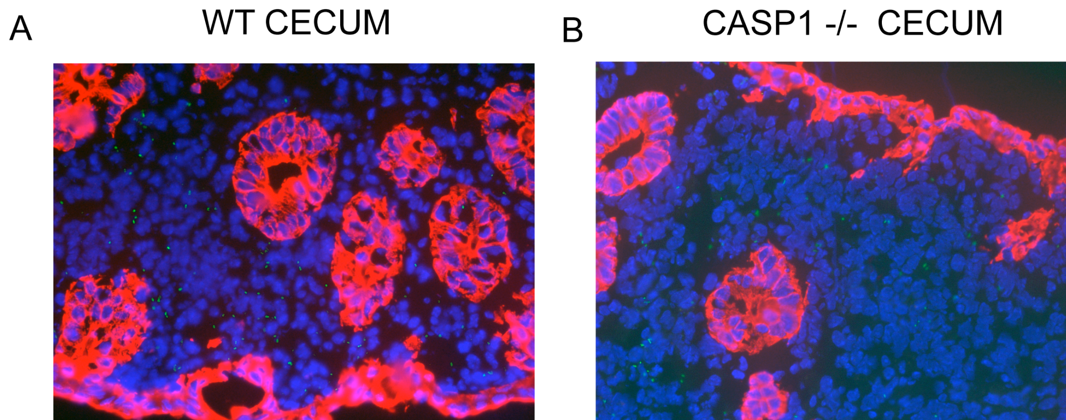

I
**
Histological Score
14
12
10
8
6
4
2
0
WT
Casp 1 -/-
S
M
L
G
E

Figure 6. Caspase-1 is required for attenuation of *flgM*-deficient *Salmonella*. C57BL/6 WT (n=9) and caspase 1 -/- (n=8) mice were infected with 1000 CFU *flgM⁻ Salmonella.* Bacterial burden in the cecum (A). Spleen (B). Liver (C). MLN (D). Representative histology of the cecum of WT mice (E) or caspase-1 -/- (F), and liver of WT mice (G) or caspase-1 -/- (H). (I) Histological scores for changes in the cecal pathology as described in Fig. 4. Figures A-D represent data from two independent experiments. Liver images (200X magnification), cecal images (100X magnification). Mann-Whitney test, * = $p<0.05$, ** = $p<0.01$, *** = $p < 0.001$

TROMA-1 STAINING



F4/80 STAINING

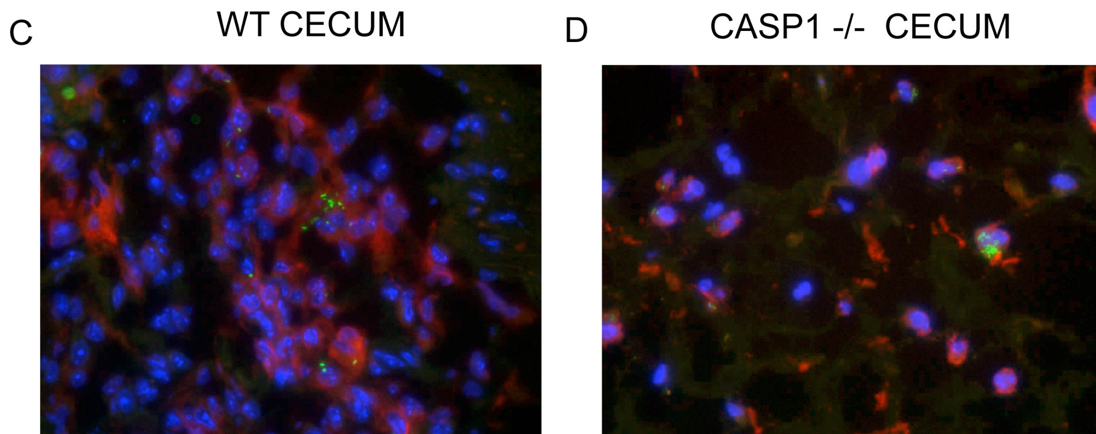
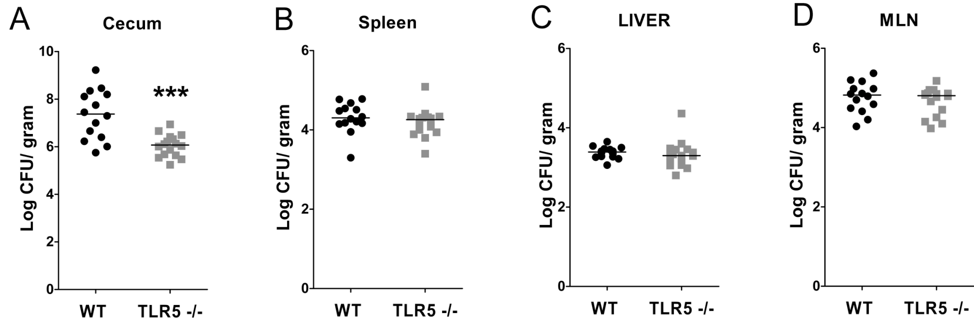
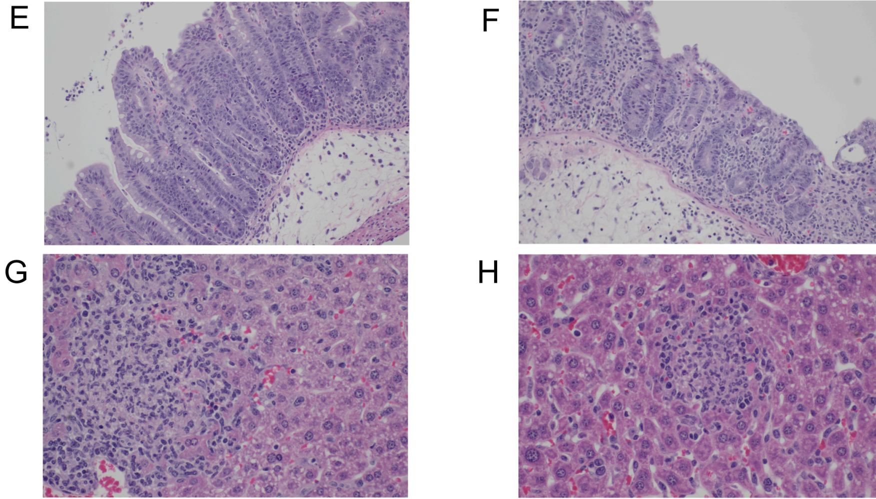


Figure 7. *Salmonella* reside within F4/80+ cells the cecum. C57BL/6 WT (n=5) (A+C) or Caspase-1 -/- (n=5) (B+D) mice were infected with 1000 cfu of *flgM*$^-$ *Salmonella* containing a stable GFP expressing plasmid. Intestinal epithelial cells were stained using TROMA (A+B), and phagocytes were stained using F4/80 (C+D) antibody. Red= TROMA-1 (A, B) and F4/80 (C, D); green = GFP (*Salmonella*). Shown are representative images from 10 mice examined.


A
Cecum
Log CFU/ gram
10
8
6
4
2
0
***
WT
TLR5 -/-
B
Spleen
Log CFU/ gram
6
4
2
0
WT
TLR5 -/-
C
LIVER
Log CFU/ gram
6
4
2
0
WT
TLR5 -/-
D
MLN
Log CFU/ gram
6
4
2
0
WT
TLR5 -/-


E
F
G
H

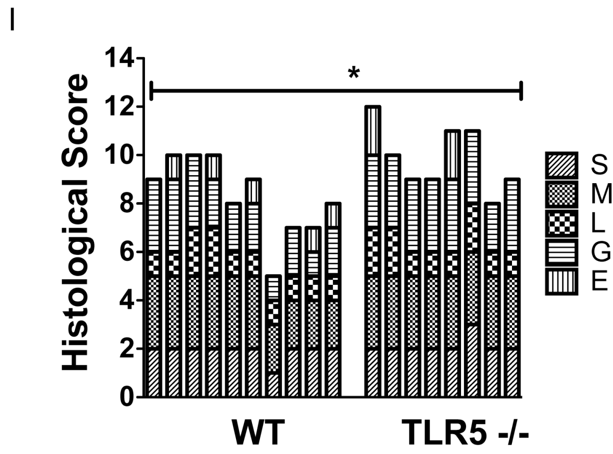
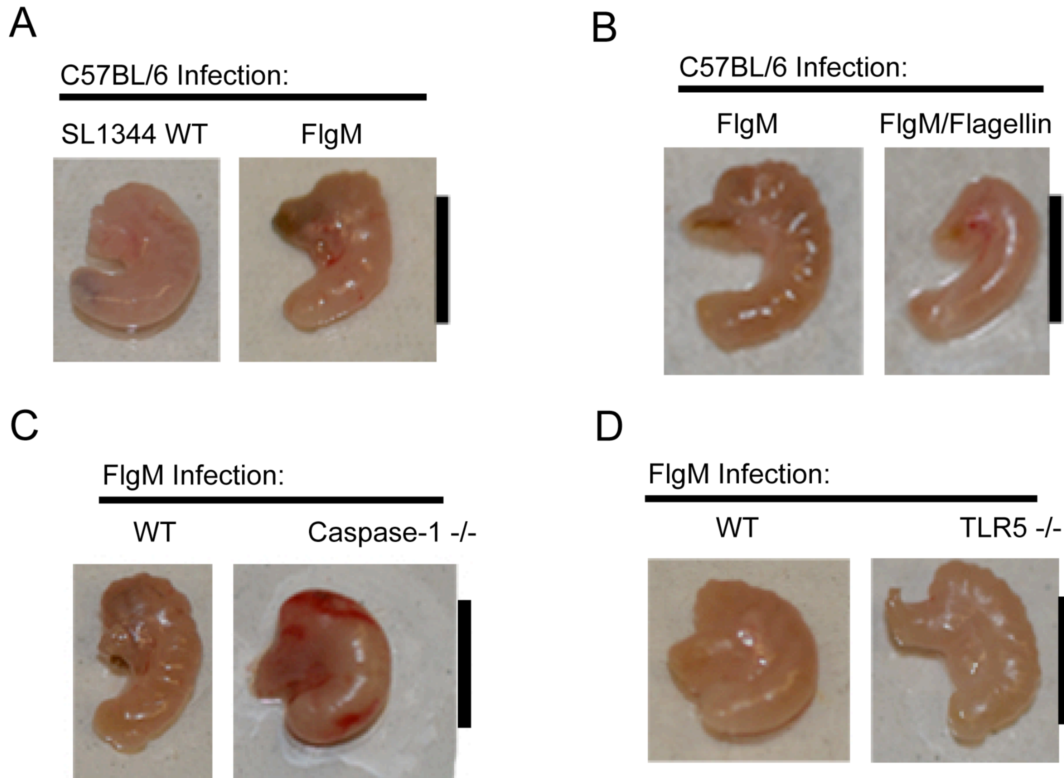
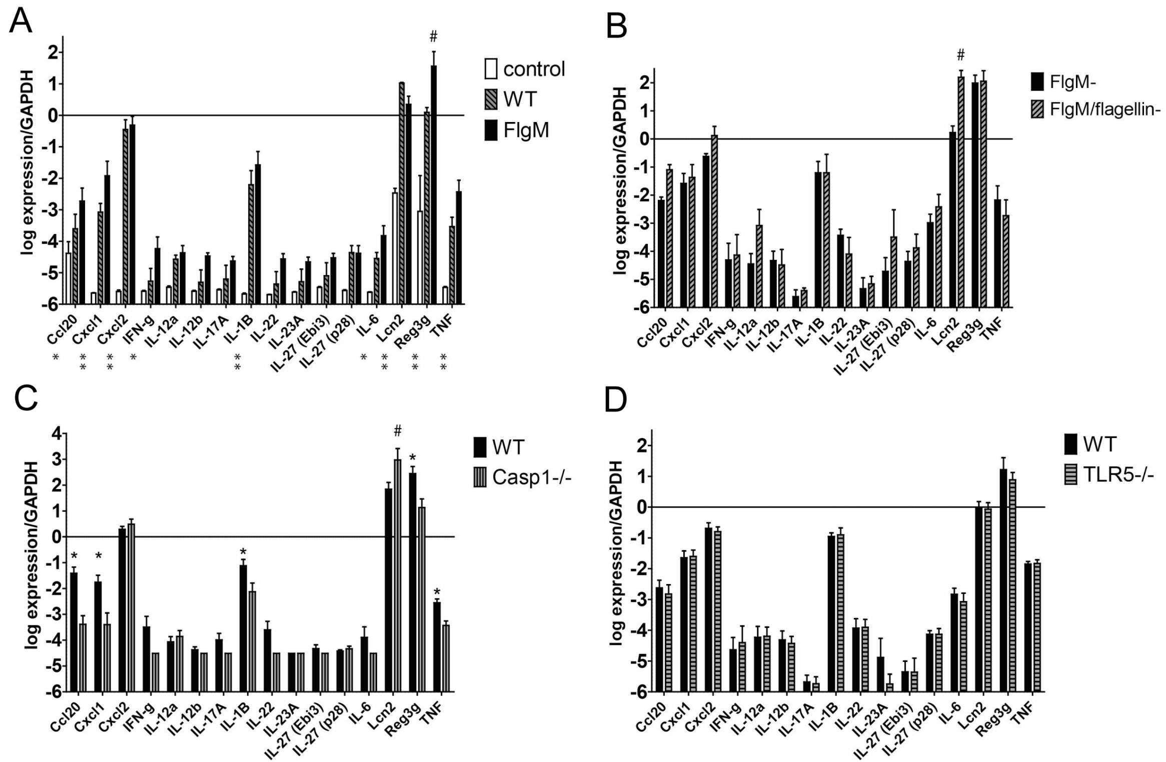

I
Histological Score
14
12
10
8
6
4
2
0
*
WT
TLR5 -/-
S
M
L
G
E

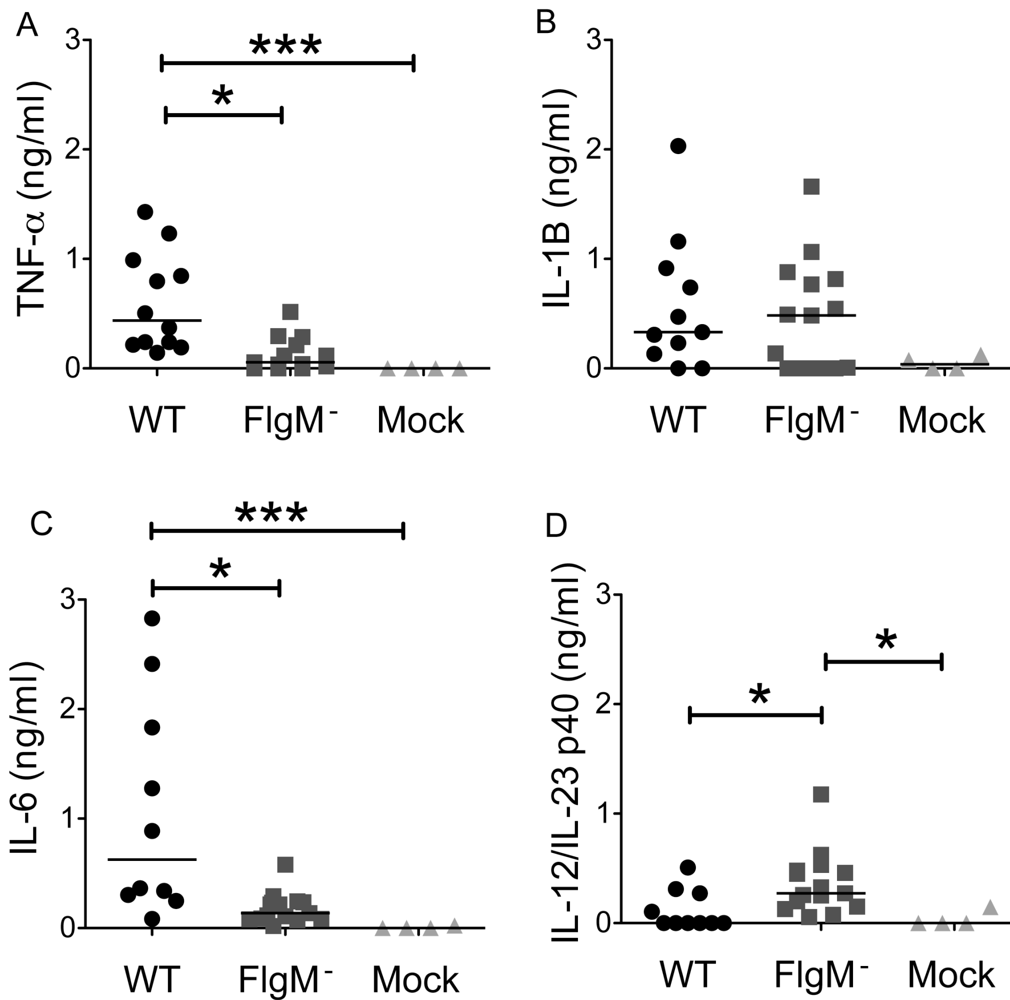
Figure 8. The innate immune receptor TLR5 is dispensable in the attenuation of *flgM*$^-$. C57BL/6 WT (n=14) and TLR5 -/- (n=16) mice were infected with 1000 CFU *flgM*$^-$ *Salmonella*. Bacterial burden in the cecum (A). Spleen (B). Liver (C). MLN (D). Representative histology of the cecum for WT mice (E) or TLR5 -/- (F), and liver of WT mice (G) or TLR5 -/- (H). Liver images (200X magnification), cecal images (100X magnification). Figures A-D represent data from three independent experiments. Mann-Whitney test * = $p<0.05$. ** = $p<0.01$.(I) Histological scores for changes in the cecal pathology as described in Fig. 4. Figures A-D represent data from three independent experiments. Mann-Whitney test *** = $p<0.001$



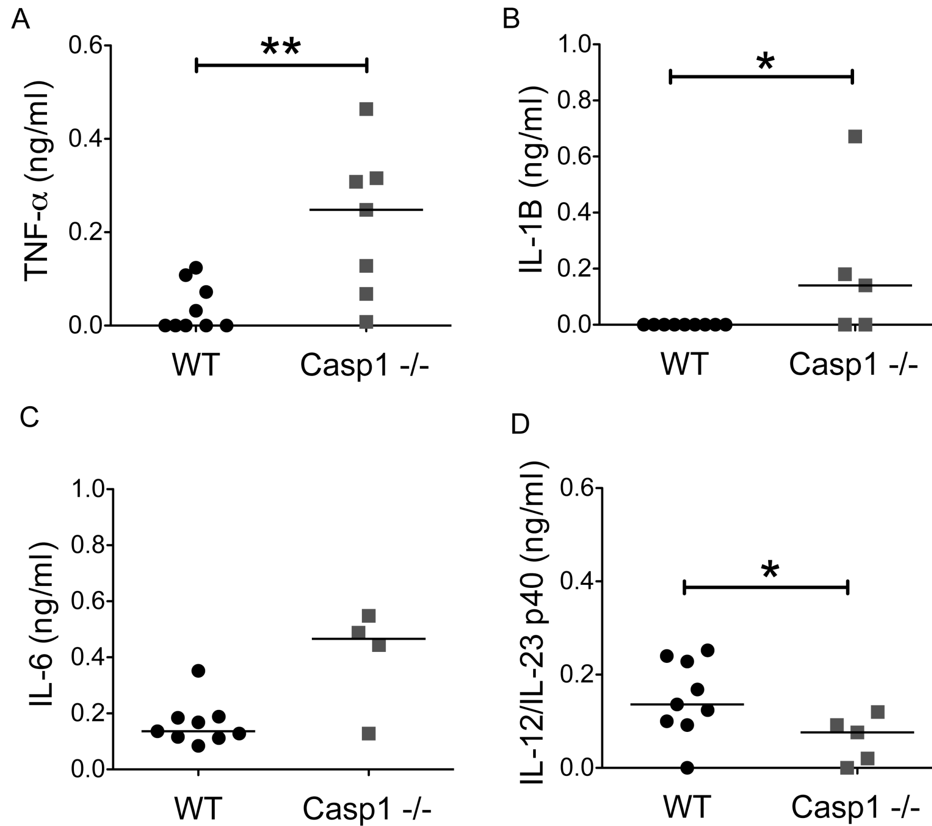
Supplementary figure 1. Gross cecum inflammation was prevalent in all *Salmonella* infected mice. Representative gross anatomy pictures of C57BL/6 WT mice infected with 1000 cfu WT SL1344 or *flgM*$^{-}$ *Salmonella* (A). Gross anatomy pictures of C57BL/6 WT mice infected with 1000 cfu *flgM*$^{-}$ or *flgM*$^{-}$ */flagellin*$^{-}$ *Salmonella* (B). Gross anatomy pictures of C57BL/6 WT or caspase-1 -/- mice infected with 1000 cfu *flgM*$^{-}$ *Salmonella* (C). Gross anatomy pictures of C57BL/6 WT or TLR5 -/- mice infected with 1000 cfu *flgM*$^{-}$ *Salmonella* (D). The bar represents 1 cm.



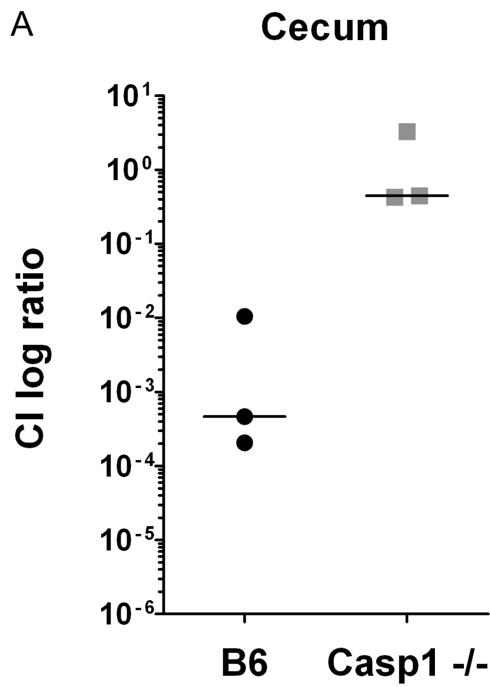
Supplementary figure 2. Flagellin and caspase-1 contribute to cytokine gene expression in the cecum. Gene expression in the cecum measured by RT-PCR for C57BL/6 mock-infected mice, or mice infected with SL1344 WT or *flgM⁻ Salmonella* (A). Genes that are significantly induced in flgM- (*) or WT and flgM- (**) Salmonella infected mice compared to mock-infected mice are designated by the asterix below the x-axis (A); genes that are significantly higher in flgM- infected mice are designated by the symbol (#) above the bars (A). Gene expression in the cecum measured by RT-PCR for C57BL/6 WT for flgM vs flgM/flagellin *Salmonella* infected mice (B). Gene expression in the cecum measured by RT-PCR for C57BL/6 WT or caspase-1 -/-mice infected with *flgM⁻ Salmonella* (C). Gene expression in the cecum measured by RT-PCR for C57BL/6 WT or TLR5 -/- mice infected with *flgM⁻ Salmonella* (D). ns = no statistical significance. Gene expression was normalized to GAPDH, and comparisons were made using the one-way ANOVA and Bonferroni's multiple comparisons test. Genes with significant differences (P < 0.05) are designated by either symbols (* or #) above the bars.

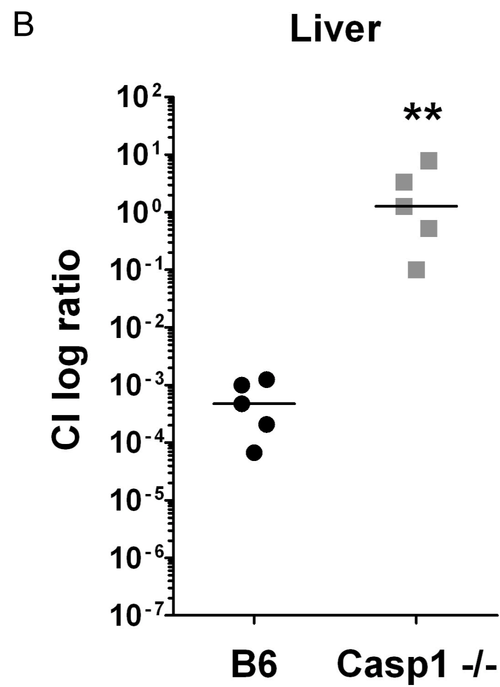


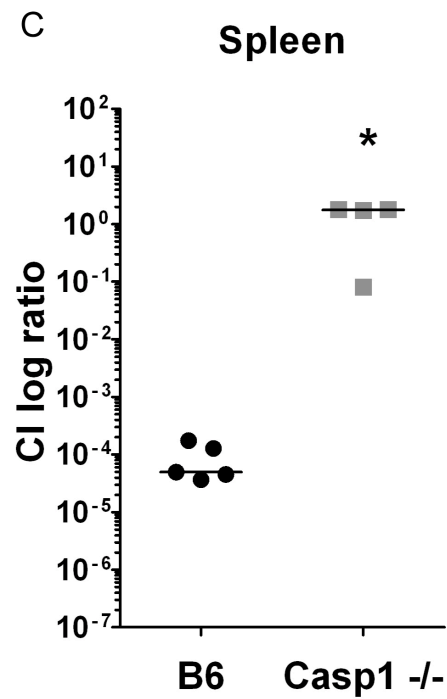
Supplementary figure 3. Increased in inflammatory cytokines in serum of WT SL1344 infected mice. ELISA measurement of serum cytokine for mice infected with 1000 cfu of SL1344 WT, *flgM⁻ Salmonella* or PBS (Mock): TNF-α (A), IL-1B (B), IL-6 (C), IL12/IL12 p40 (D). Figures A-D represent data from three independent experiments (WT n= 12; *flgM⁻* n=15; mock n=4). Statistical analysis with one-way ANOVA using the Kruskal-Wallis test and Dunn's multiple comparisons test, * = p<0.05. *** = p<0.001.

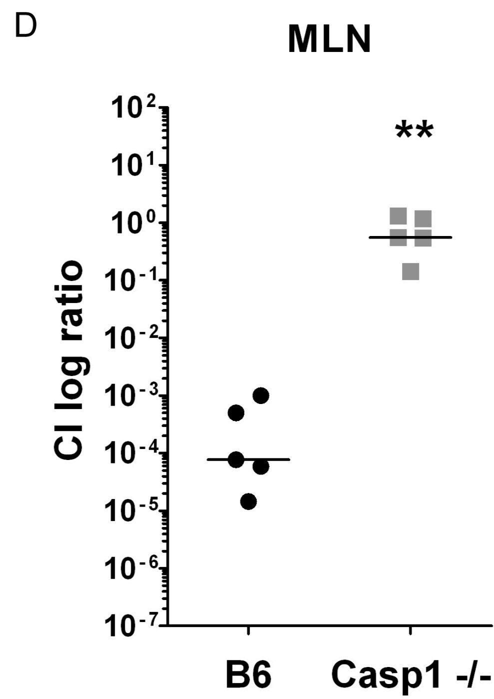


Supplementary figure 4. Increased in inflammatory cytokines in serum of Caspase-1 -/- infected mice. C57BL/6 WT or Caspase-1 -/- mice were infected with 1000 cfu of *flgM⁻ Salmonella*. ELISA measurement of serum cytokine for TNF-α (A), IL-1B (B), IL-6 (C), IL-12/IL23 p40 (D). Figures A-D represent data from two independent experiments (WT n=9; caspase-1 -/- n= 8). ELISAs were not performed for all cytokines due to poor serum yield. Mann-Whitney test * = $p<0.05$. ** = $p<0.01$.

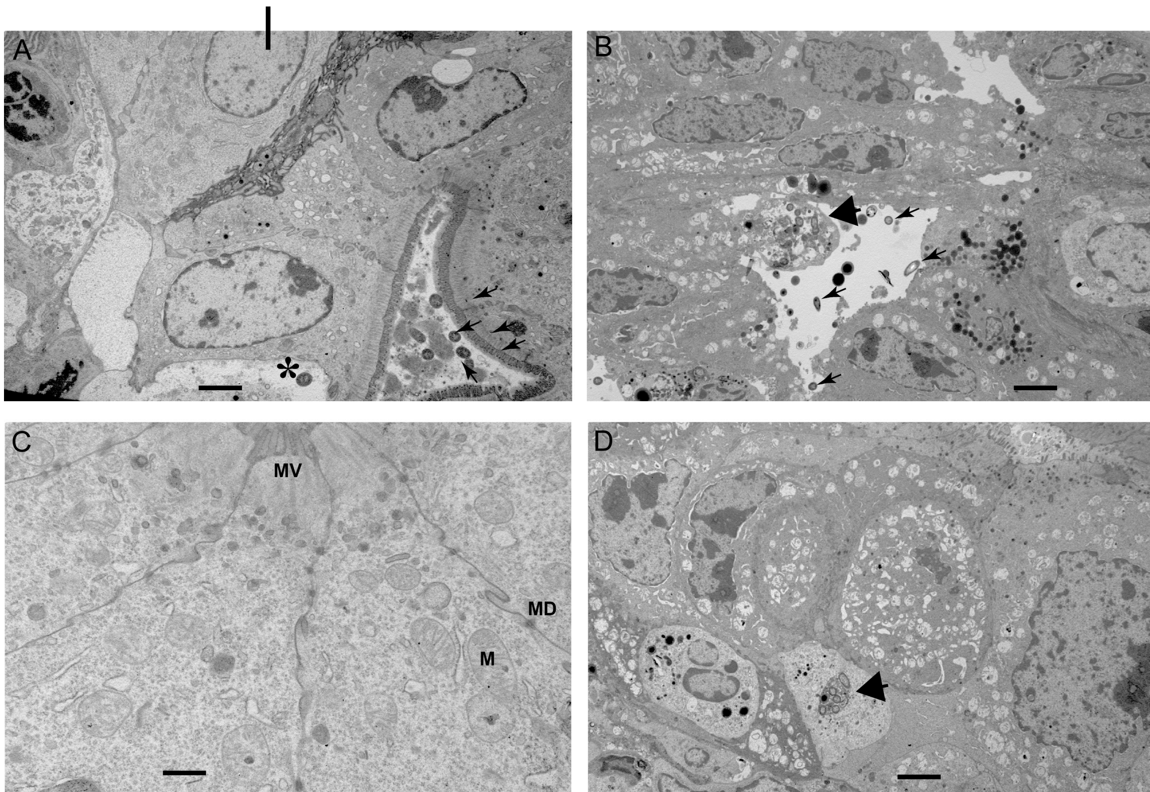

A
Cecum
CI log ratio
10^1
10^0
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
B6
Casp1 -/-


B
Liver
CI log ratio
**
10^2
10^1
10^0
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
10^{-7}
B6
Casp1 -/-


C
Spleen
CI log ratio
*
10^2
10^1
10^0
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
10^{-7}
B6
Casp1 -/-

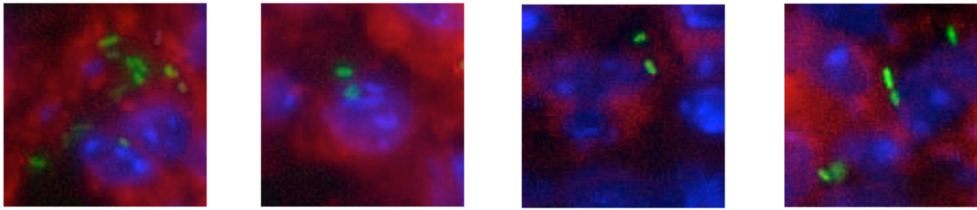

D
MLN
CI log ratio
**
10^2
10^1
10^0
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
10^{-7}
B6
Casp1 -/-

Supplementary figure 5. Decreased virulence of *flgM- Salmonella* is dependent on caspase-1. *In vivo* competitive index assays where performed by infecting C57BL/6 WT (n=5) or caspase-1 -/- (n=5) mice with an inoculum containing 500 cfu WT SL1344 and 500 cfu *flgM- Salmonella*. CFU for the WT and *flgM-* bacteria were enumerated, and the log ratios (*flgM-*/WT) were plotted for cecum (A), liver (B), spleen (C), MLN (D). Log ratio was used to demonstrate increases or decreases of virulence between SL1344 WT and *flgM-Salmonella.* Mann-Whitney test * = $p<0.05$. ** = $p<0.01$.

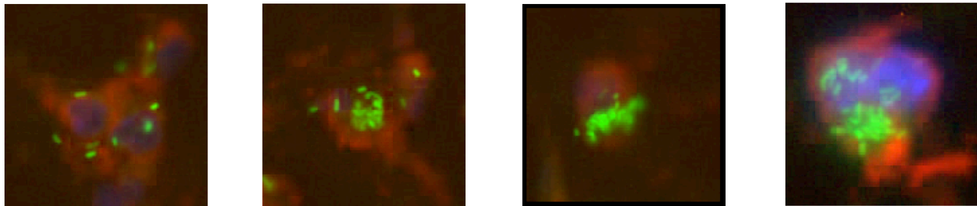


Supplementary figure 6. Ultrastructural localization of *flgM*- *Salmonella* in the epithelium. Ceca from C57BL/6 WT (A,C) and Casp1-/- (B,D) mice were analyzed for electron microscopy. *Salmonella*-like bacteria were detected in the lumina for both mice (arrows, A and B), and in the cytosol of some epithelial cells in Casp1-/- mice (arrowheads, B). Rare bacteria were detected in mechanically disrupted epithelial cells of WT mice (asterix, A) and likely represent artifact. The epithelium was well preserved in the WT mice (microvilli - M, macula densa - MD, and mitochondria - M). Focally, bacteria were seen in intracellular vesicles within cells in the epithelial layer (arrowhead, D), possibly representing *Salmonella* in leukocyte vacuoles.

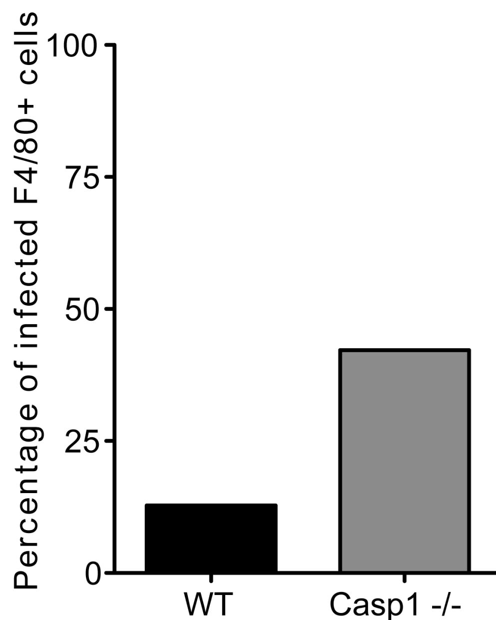
A WT infection:



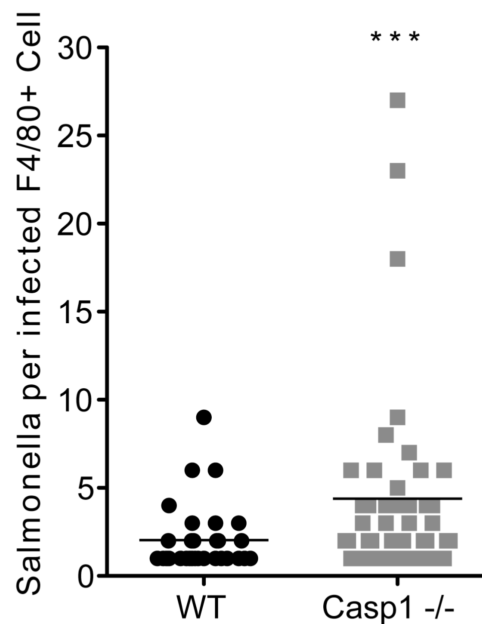
B Casp1 -/- infection:



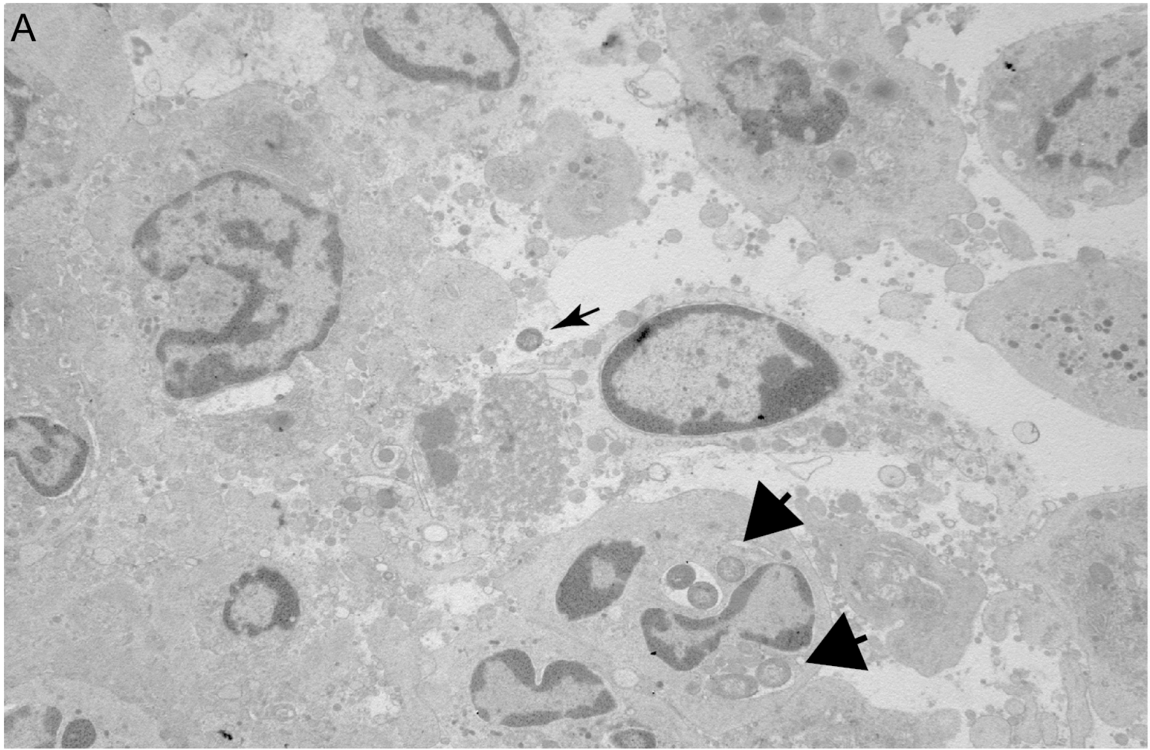
C

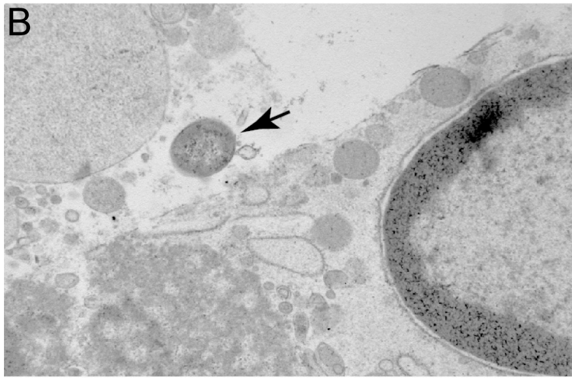


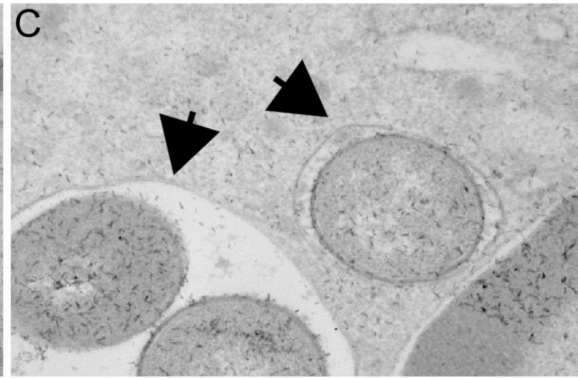
D



Supplementary figure 7. Caspase-1 controls intracellular *Salmonella*. C57BL/6 WT or Caspase-1 -/- mice were infected with 1000 cfu of *flgM*⁻ *Salmonella* containing a stable GFP expressing plasmid. Frozen cecal tissue was stained using F4/80 antibody in WT (A) and caspase-1 -/- (B). The percentage of F4/80+ cells associated with GFP+ bacteria (C) and the number of GFP+ *Salmonella* associated with each F4/80+ cell (D) were quantified for ten 40X-objective high power fields from WT and caspase-1 -/- mice. Mann-Whitney test. *** = p<0.001.


A


B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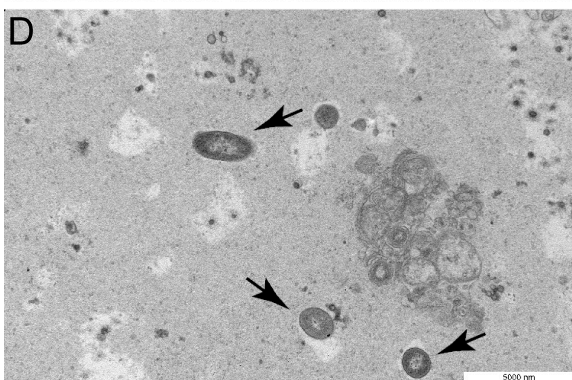
D
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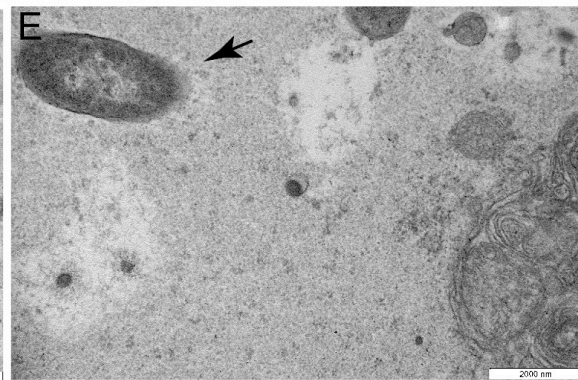
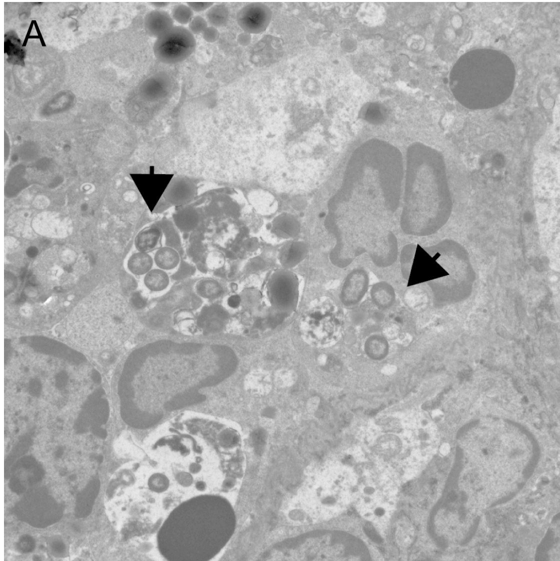

E
2000 nm

Supplementary figure 8. Ultrastructural localization of *flgM- Salmonella* in the lamina propria and submucosa of WT mice. Within the lamina propria and submucosa of C57BL/6 WT mice, Salmonella-like bacteria were detected outside of cells (arrows, A) and inside of cells (arrowheads, A). Examination at higher magnification revealed that the extracellular bacteria (arrows; B,D,E) were adjacent to degenerating cells and cellular debris. The intracellular bacteria were infrequent, and present within simple vesicles contained by a single lipid bilayer (arrowheads, C).



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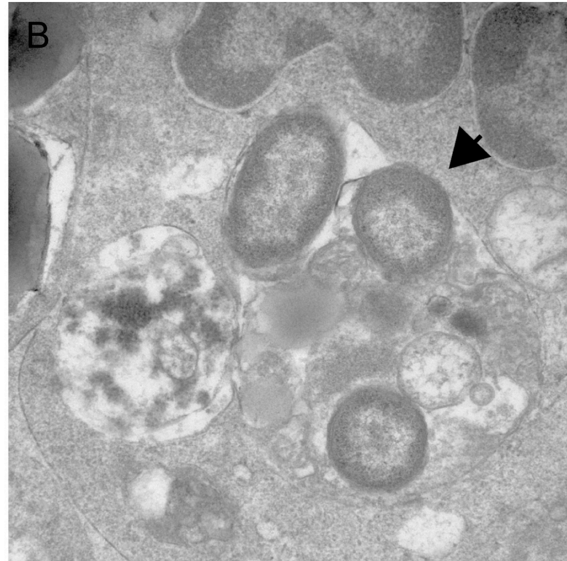
UWMC EM Lab



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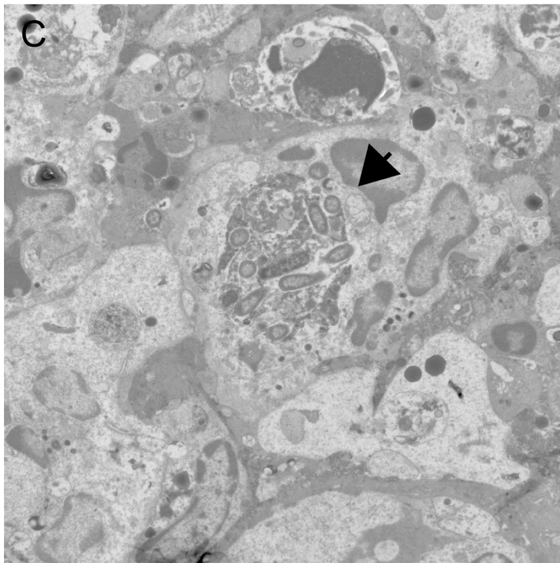

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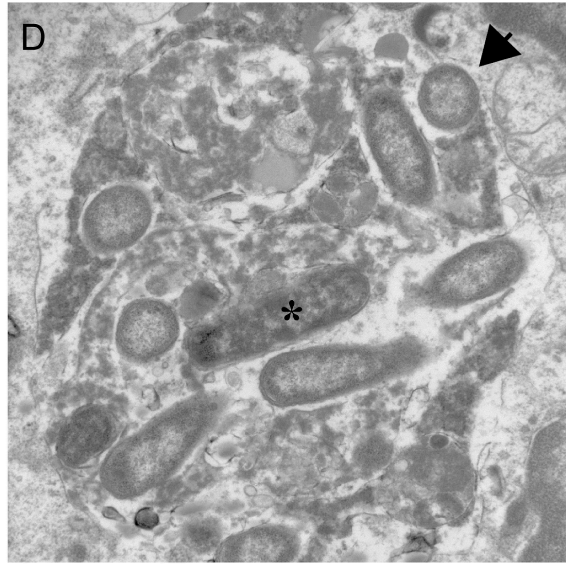

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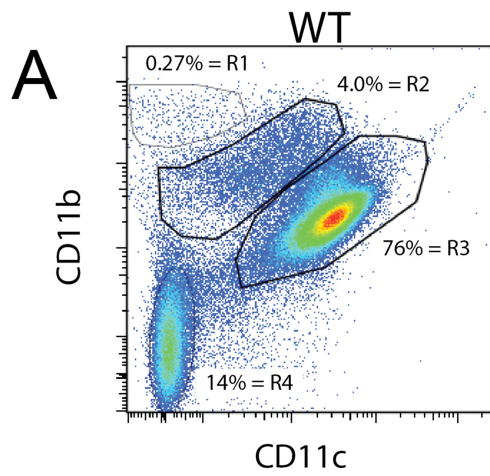
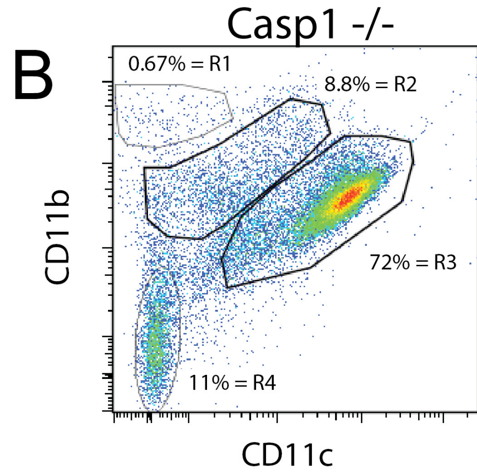
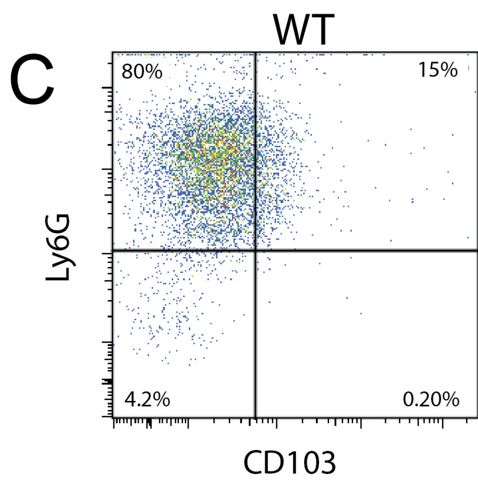
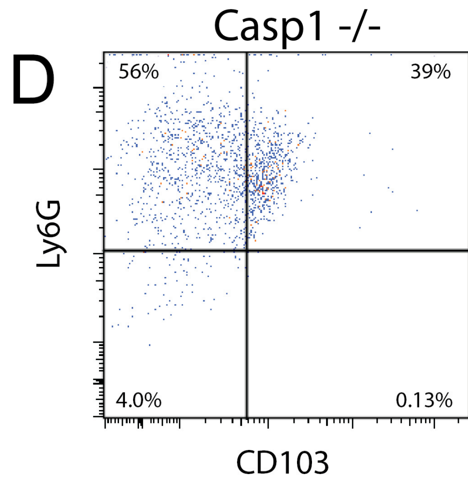
UWMC EM Lab

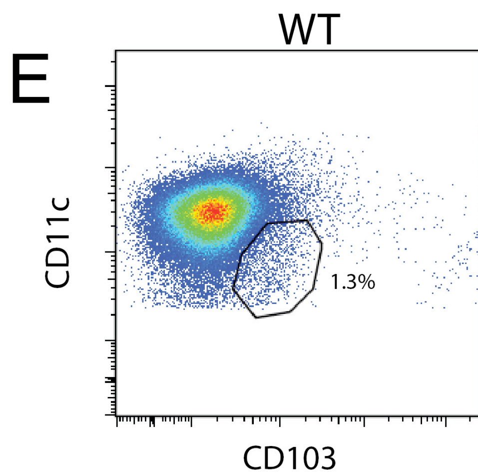
Supplementary figure 9. Ultrastructural localization of *flgM- Salmonella* in the lamina propria and submucosa of Casp1-/- mice. Within the lamina propria and submucosa of C57BL/6 Casp1-/- mice (A-D), Salmonella-like bacteria were detected predominantly inside of cells (arrowheads), and within complex heterogenous vesicles. Some of the bacteria showed loss of integrity of the cell wall (asterix, D).

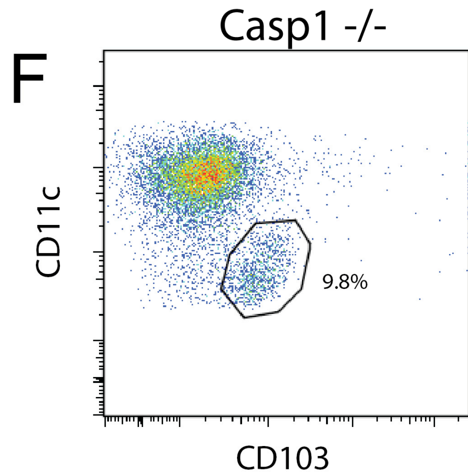

WT
A
0.27% = R1
4.0% = R2
76% = R3
14% = R4
CD11b
CD11c


Casp1 -/-
B
0.67% = R1
8.8% = R2
72% = R3
11% = R4
CD11b
CD11c

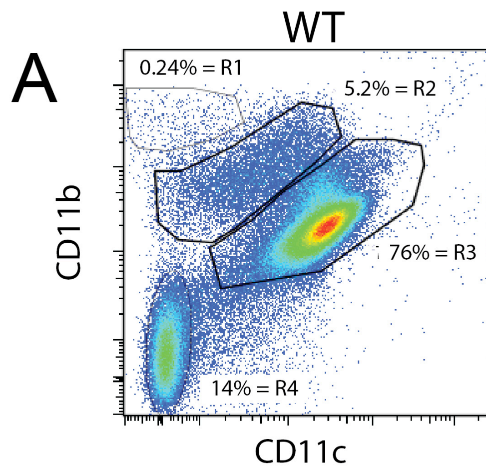

WT
C
80%
15%
4.2%
0.20%
Ly6G
CD103

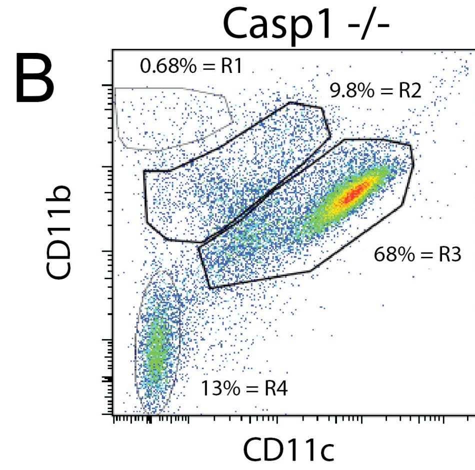

Casp1 -/-
D
56%
39%
4.0%
0.13%
Ly6G
CD103

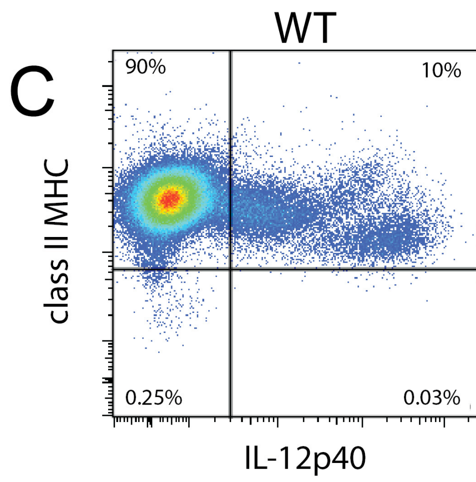

WT
E
1.3%
CD11c
CD103

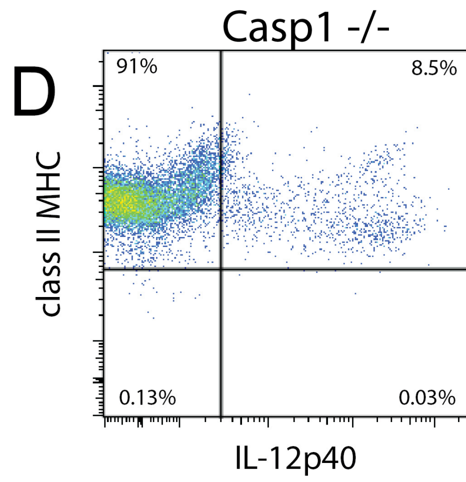

Casp1 -/-
F
9.8%
CD11c
CD103

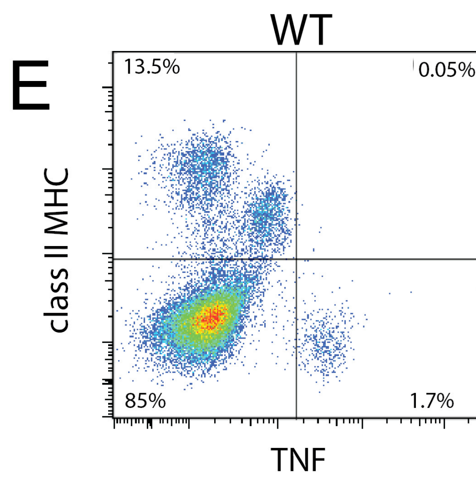
Supplementary figure 10. Caspase-1 regulates CD103+ cells in the lamina propria of *Salmonella* infected mice. Lamina propria cells were prepared from the large intestine of C57BL/6 WT or Caspase-1 -/- mice that were infected for 5 days with 1000 CFU of *flgM*$^-$ *Salmonella*. (A,B) CD11b and CD11c staining of live cells gated by forward and side scatter defined four subpopulations (R1, R2, R3, and R4) in the C57BL/6 WT (A) and caspase-1 -/- mice (B). Lamina propria cell R2 population staining for Ly6G and CD103 in C57BL/6 WT (C) or caspase-1 -/- (D). Lamina propria cell R3 population staining for CD11c and CD103 in C57BL/6 WT (E) and Caspase-1 -/- (F). Shown are representative plots from four independent mice (2 WT and 2 Caspase-1 -/-).

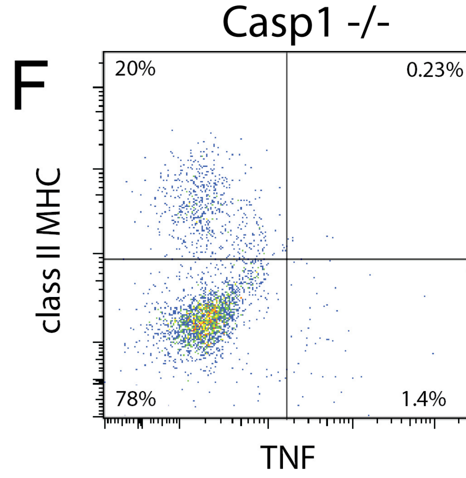

A
WT
0.24% = R1
5.2% = R2
76% = R3
14% = R4
CD11b
CD11c


B
Casp1 -/-
0.68% = R1
9.8% = R2
68% = R3
13% = R4
CD11b
CD11c

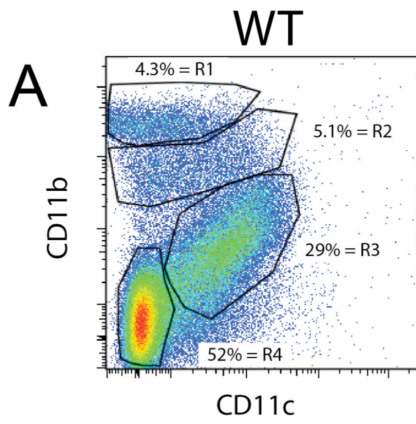

C
WT
90%
10%
0.25%
0.03%
class II MHC
IL-12p40

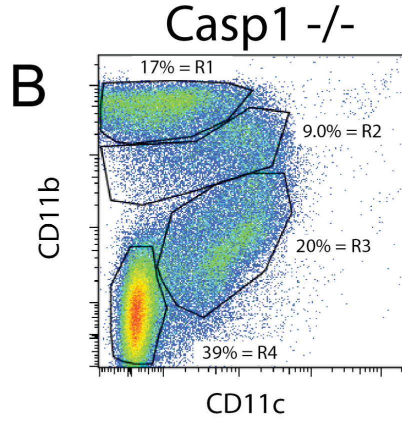

D
Casp1 -/-
91%
8.5%
0.13%
0.03%
class II MHC
IL-12p40

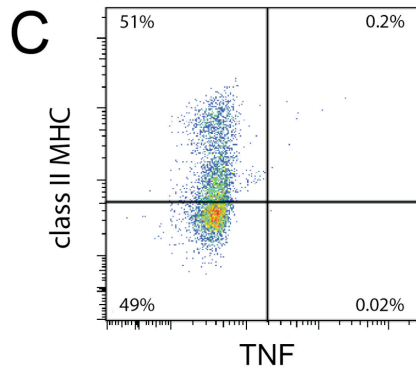

E
WT
13.5%
0.05%
85%
1.7%
class II MHC
TNF

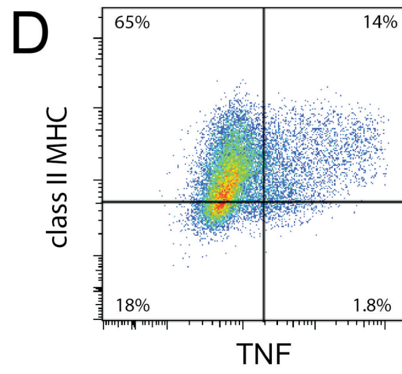

F
Casp1 -/-
20%
0.23%
78%
1.4%
class II MHC
TNF

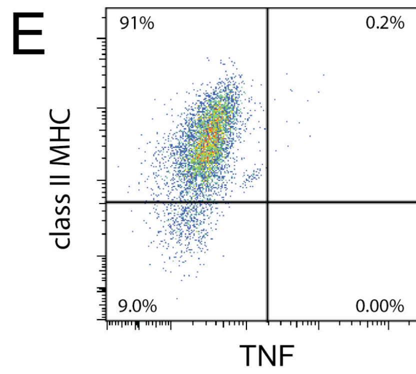
Supplementary figure 11. Cytokine production in the lamina propria cells of *flgM- Salmonella* infected WT and caspase-1-/- mice Lamina propria cells were prepared from the large intestine of C57BL/6 WT or Caspase-1 -/- mice that were infected for 5 days with 1000 CFU of *flgM⁻ Salmonella*, and incubated for 4 hr in the presence of brefeldin A to detect spontaneous cytokine production. (A,B) CD11b and CD11c staining of live cells gated by forward and side scatter defined four subpopulations (R1, R2, R3, and R4) in the C57BL/6 WT (A) and caspase-1 -/- mice (B). Class II MHC and IL-12p40 staining in the R3 population from WT (C) and caspase-1-/- (D) mice. Class II MHC and TNF staining in the R4 population from WT (E) and caspase-1-/- (F) mice.

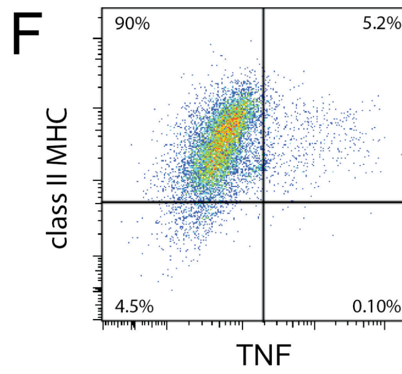

WT
A
4.3% = R1
5.1% = R2
29% = R3
52% = R4
CD11b
CD11c

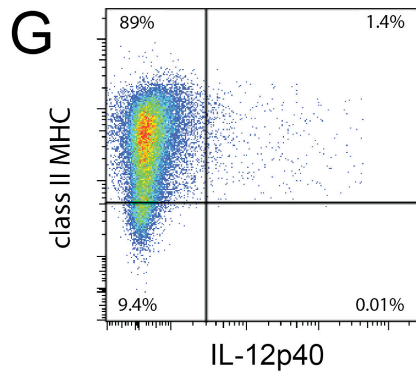

Casp1 -/-
B
17% = R1
9.0% = R2
20% = R3
39% = R4
CD11b
CD11c

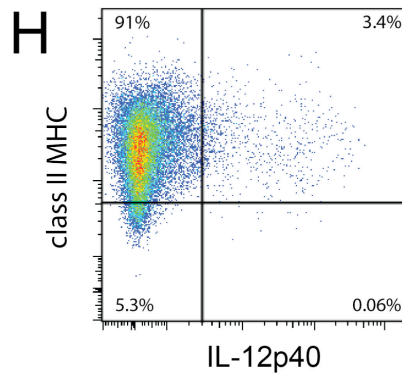

C
51%
0.2%
49%
0.02%
class II MHC
TNF


D
65%
14%
18%
1.8%
class II MHC
TNF

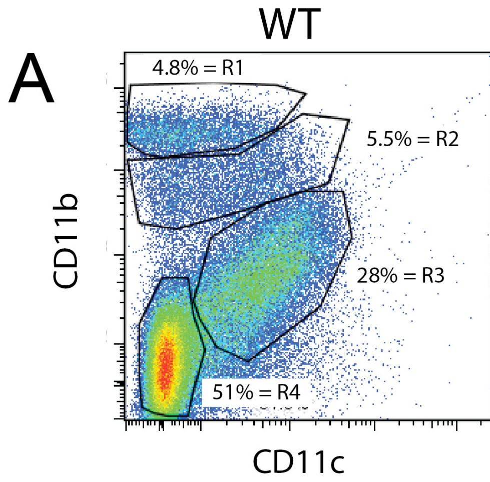

E
91%
0.2%
9.0%
0.00%
class II MHC
TNF

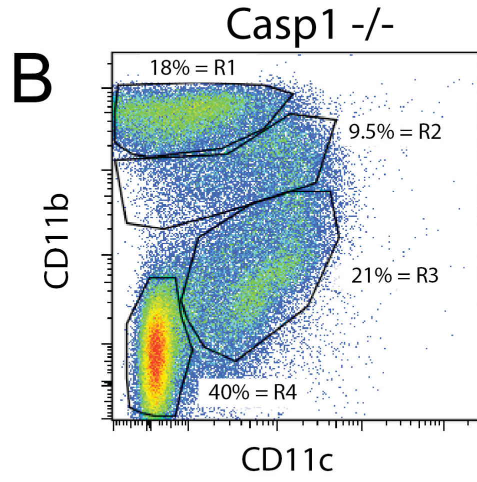

F
90%
5.2%
4.5%
0.10%
class II MHC
TNF

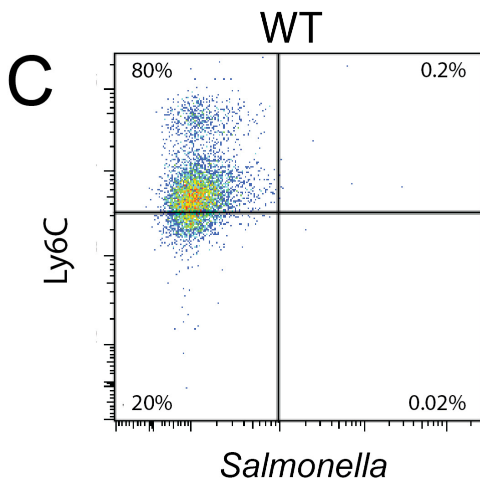

G
89%
1.4%
9.4%
0.01%
class II MHC
IL-12p40

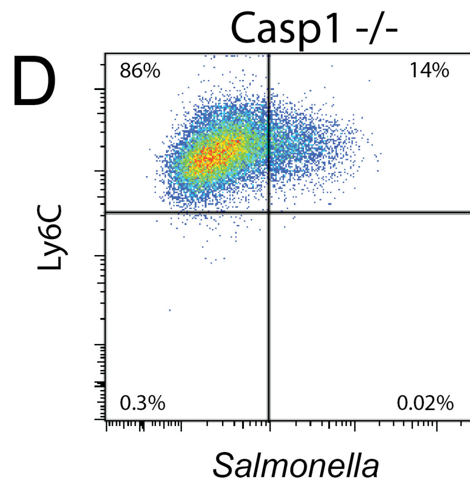

H
91%
3.4%
5.3%
0.06%
class II MHC
IL-12p40

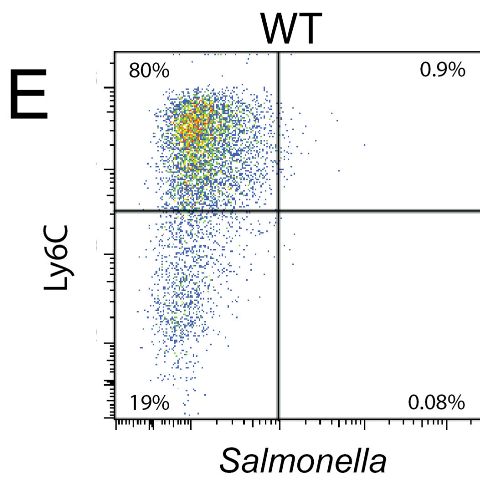
Supplementary figure 12. Cytokine production by spleen cells from *flgM-* *Salmonella* infected WT and caspase-1-/- mice. Spleen cells were prepared from C57BL/6 WT or Caspase-1 -/- mice that were infected for 5 days with 1000 CFU of *flgM*⁻ *Salmonella*, and incubated for 4 hr in the presence of brefeldin A to detect spontaneous cytokine production. (A,B) CD11b and CD11c staining of live cells gated by forward and side scatter defined four subpopulations (R1, R2, R3, and R4) in the C57BL/6 WT (A) and caspase-1 -/- mice (B). Class II MHC and TNF staining for C57BL/6 WT mice R1 (C) and R2 (E) populations, and the caspase-1-/- mice R1 (D) and R2 (F) populations. Class II MHC and IL-12p40 in the R3 population from C57BL/6 WT (G) and Caspase-1 -/- (H) mice.

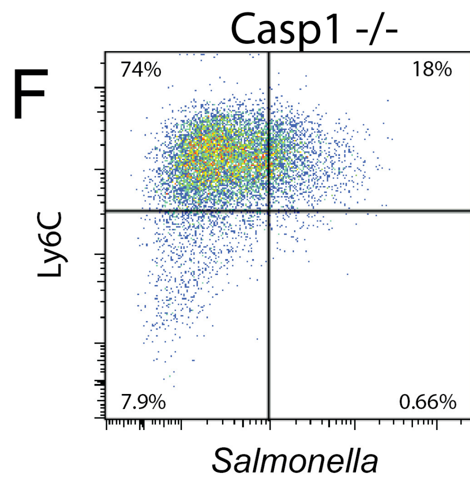

WT
A
4.8% = R1
5.5% = R2
28% = R3
51% = R4
CD11b
CD11c


Casp1 -/-
B
18% = R1
9.5% = R2
21% = R3
40% = R4
CD11b
CD11c

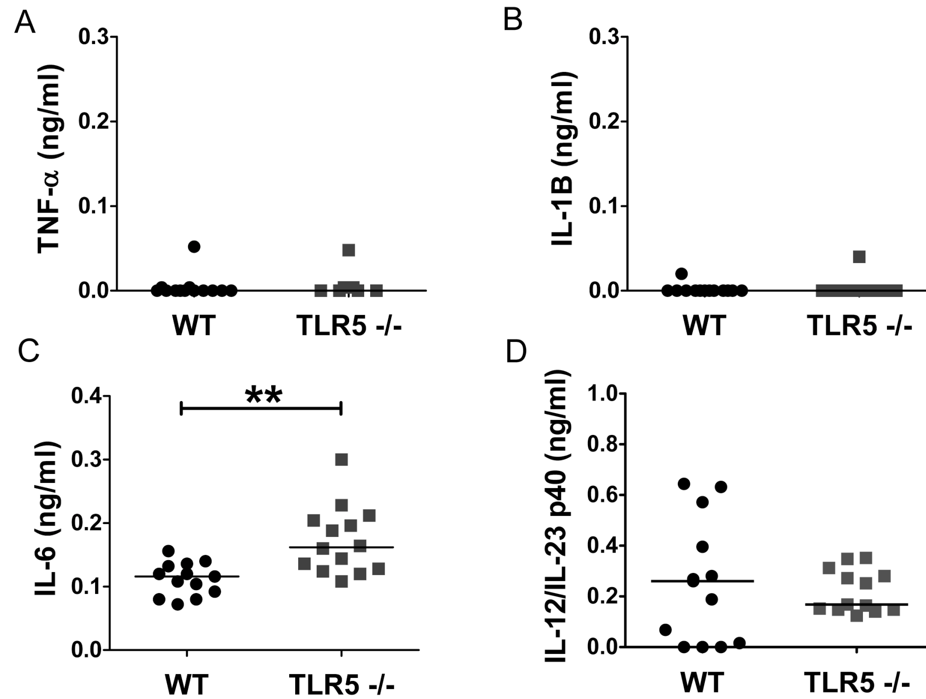

WT
C
80%
0.2%
20%
0.02%
Ly6C
Salmonella


Casp1 -/-
D
86%
14%
0.3%
0.02%
Ly6C
Salmonella


WT
E
80%
0.9%
19%
0.08%
Ly6C
Salmonella

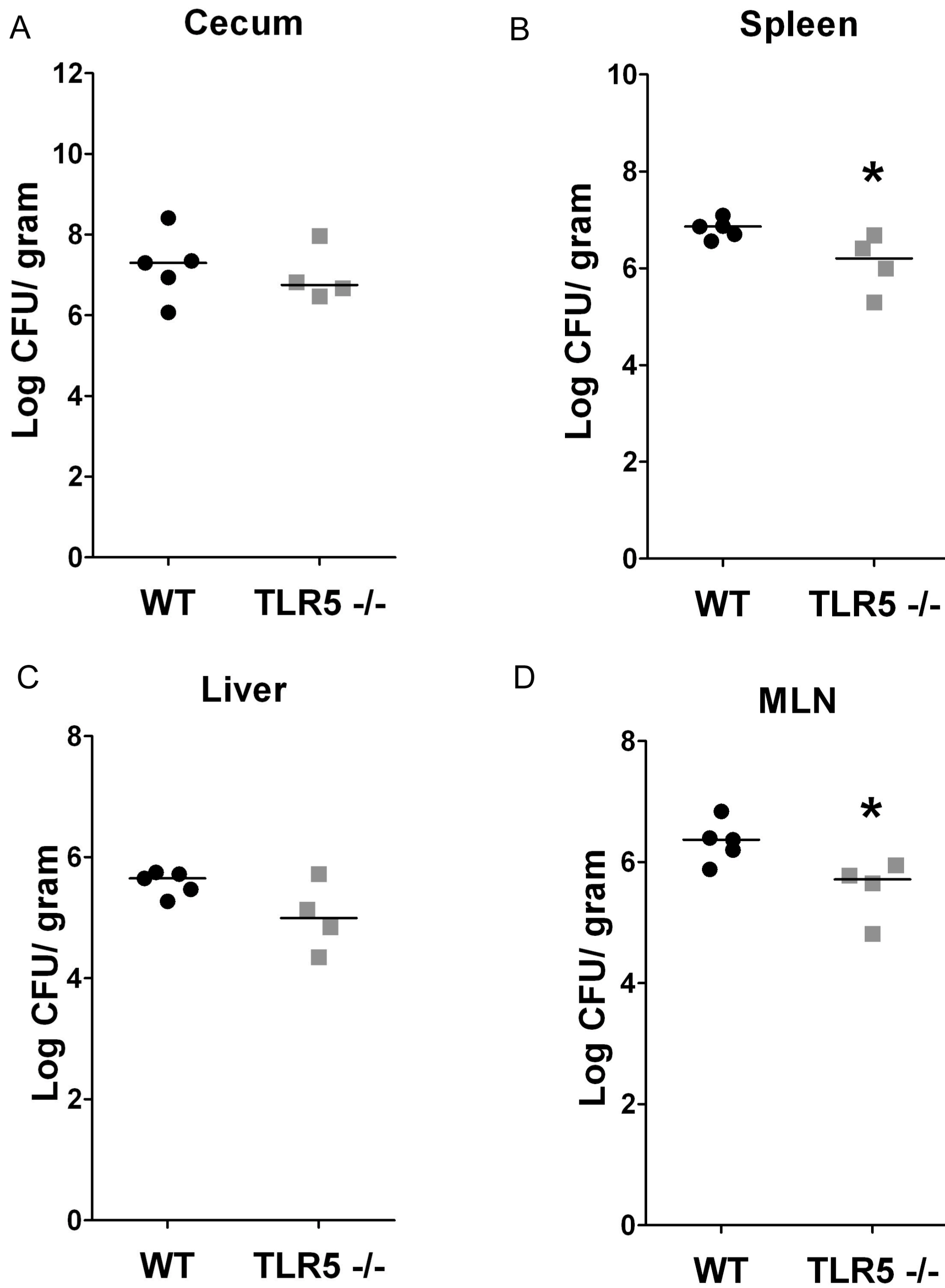

Casp1 -/-
F
74%
18%
7.9%
0.66%
Ly6C
Salmonella

Supplementary figure 13. *Salmonella* reside in phagocytes in caspase-1 deficient mice. Spleen cells were prepared from C57BL/6 WT or Caspase-1 -/- mice that were infected for 5 days with 1000 CFU of *flgM*[-] *Salmonella*, (A,B) CD11b and CD11c staining of live cells gated by forward and side scatter defined four subpopulations (R1, R2, R3, and R4) in the C57BL/6 WT (A) and caspase-1 -/- mice (B). Ly6C and *Salmonella* staining in C57BL/6 WT R1 (C) and R2 (E) populations, and caspase-1-/- R1 (D) and R2 (F) populations.

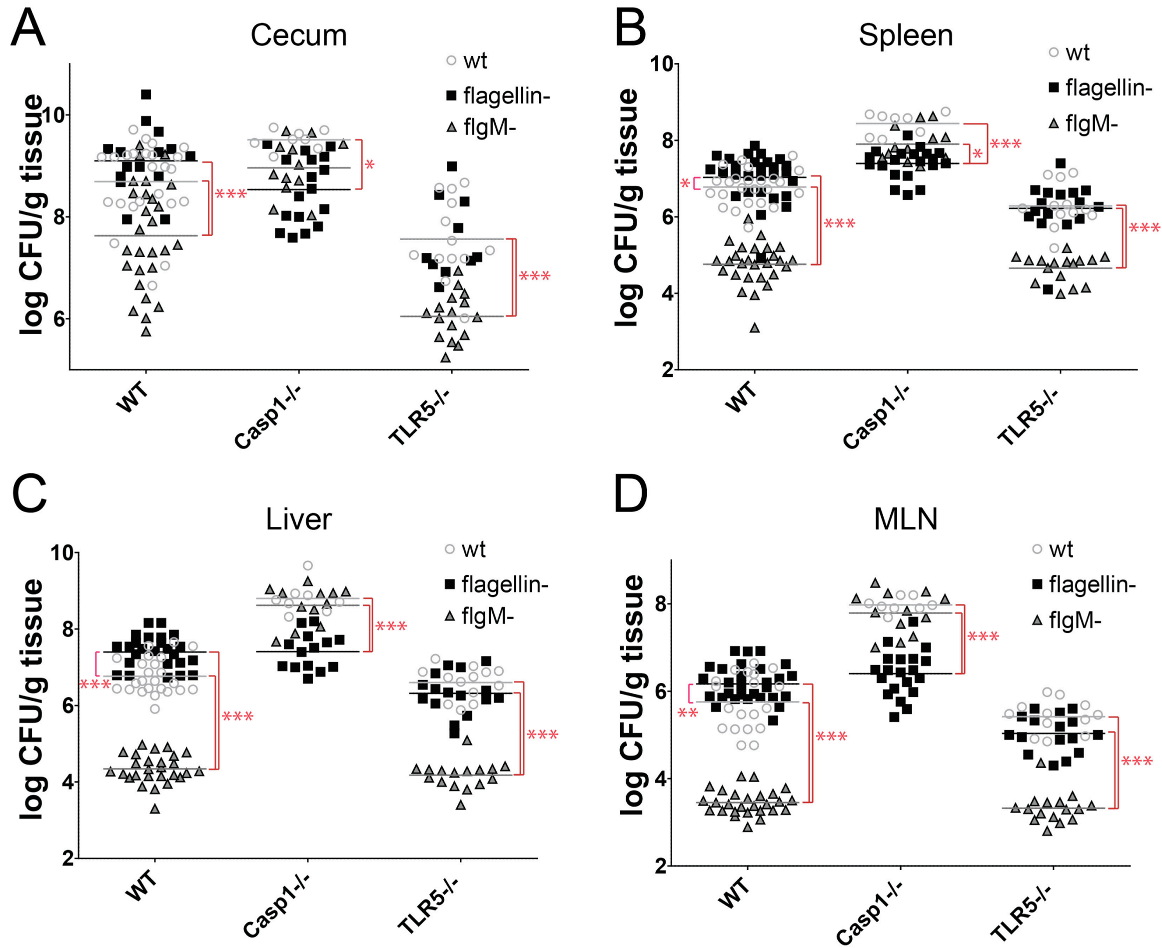


Supplementary figure 14. TLR5 is dispensable in inflammatory responses against *flgM⁻ Salmonella*. C57BL/6 WT (n=14) and TLR5 -/- (n=16) mice infected with 1000 cfu of *flgM⁻ Salmonella.* ELISA measurement of serum cytokine for TNF (A), IL-1B (B), IL-6 (C), IL-12 p40 (D). Figures A-D are the combined data of three independent experiments.

flgM/flagellin- Salmonella infection:



Supplementary figure 15. TLR5 promotes cecal colonization of *flgM-* *Salmonella* independent of *Salmonella* flagellin. Bacterial burden WT C57BL/6 (n=5) and TLR5 -/- (n=4) mice infected with 1000 cfu *flgM*/flagellin^{-} *Salmonella* in the cecum (A), spleen (B), liver (C), MLN (D). Mann-Whitney test * = $p<0.05$.



Supplementary figure 16. Composite analysis of Salmonella WT, flagellin- and flgM- infections in WT, caspase-1-/- and TLR5-/- mice. We combined the CFU data for all experiments using WT, flagellin deficient and *flgM- S*. Typhimurium SL1344, and the data was analyzed using two-way ANOVA and Bonferroni's multiple comparisons post tests. * = $P < 0.05$, ** = $P < 0.01$, and *** = $P < 0.001$. Cecum (A), Spleen (B), Liver (C), and MLN (D).

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Chapter 3: Positive and negative regulation of *Salmonella* infection by Naip5

Abstract

Salmonella enterica serovar Typhimurium is a leading cause of gastroenteritis worldwide and a deadly pathogen in children, immunocompromised and elderly. *Salmonella*'s flagellin induces an innate immune response that is dependent on an inflammasome composed of Casp1, Nlrc4, and Naip5. We hypothesized that the Naip5/Nlrc4 inflammasome has distinct roles during systemic and mucosal detection of flagellin. To test this hypothesis, we used wildtype, flagellin deficient and flagellin overproducing *Salmonella* to assess the contribution of each inflammasome component during systemic and mucosal infection. We demonstrate that during systemic infection, *Salmonella* efficiently evades flagellin detection. During mucosal infections, Casp1 and Nlrc4 are required for flagellin detection and host protection. In contrast, Naip5 has a more complex function. Similar to Nlrc4 and Casp1, loss of Naip5 abrogates *Salmonella* flagellin detection *in vivo*, and increases host susceptibility to infection. In addition, loss of Naip5 leads to protection against *Salmonella* infection, which is independent of flagellin expression by *Salmonella*. Thus Naip5 detection of *Salmonella*'s flagellin protects against infection, and Naip5 promotes *Salmonella* infection through an undefined mechanism, possibly involving

interactions with the commensal microbiota. Our studies illuminate the complexity of mucosal infections, where the multitude of interactions between microbiota, pathogens and the innate immune system shape host defenses.

Introduction

Salmonella is the causative agent in Salmonellosis and is a leading cause of gastrointestinal bacterial infections worldwide (cite CDC). Consumption of contaminated food is responsible for most cases of *Salmonella* infection, which is characterized by nausea, vomiting, diarrhea and abdominal clamps. During the initial phase of the infection, the bacteria travel to the intestine where they transverse the intestinal epithelial barrier, leading to the activation of the innate immune system. Innate immune recognition of *Salmonella* is mediated by evolutionarily conserved receptors capable of sensing conserved bacterial structures that promote inflammatory and immune responses [18]. *Salmonella's* flagellin is one of these conserved bacterial structure that elicit strong immune responses by activating two independent pathways i) Toll-like receptor 5 (TLR5) and ii) caspase-1 dependent inflammasome. Bacterial flagellin has been studied for decades due to its importance in motility. Flagellin based motility and chemotaxis are important for inducing acute colitis, and competing with other microbiota for nutrients in the inflamed gut [52]; [60]. In a previous study, we demonstrated that *Salmonella's* flagellin strongly activates the innate immune system *in vivo*. Using a streptomycin pretreatment infection model, we demonstrated that

TLR5 deficiency paradoxically protects against *Salmonella* infection and enhances inflammation in the cecum [89]. The TLR5 phenotype was independent of flagellin expression by *Salmonella* and presumably resulted from interactions between TLR5 and non-*Salmonella* flagellin molecules derived from the gut microbiota. In contrast, caspase-1 inflammasome mediated protection required *Salmonella* flagellin. Activation of the inflammasome by Salmonella's flagellin also protected the gut from inflammation [89].

The caspase-1 inflammasome detects a conserved site in the carboxy terminus of flagellin by using the intracellular receptors Naip5 and Naip6 [27,62]. The binding of flagellin to these receptors leads to the formation of a complex that includes Nlrc4 and caspase-1 [27,62]. This results in activation of caspase-1 through autocleavage. Nlrc4 contains a caspase activating region domain (CARD), can associate with caspase-1 in the absence of Asc and trigger cell death [29]. Recruitment of Asc to the Nlrc4 inflammasome leads to the efficient processing of IL-1B and IL18 [29]. During oral infection with *Salmonella* caspase-1 protects mice via IL-1B and IL-18 production, which is mediated by the Nlrc4 and Nlrp3 inflammasomes [29,30,65]. However, during intraperitoneal *Salmonella* infection the Nlrc4 inflammasome controls infection of mice via pyroptosis and independent of IL-1B and IL-18 [66]. The specific roles of the components that mediate caspase-1 dependent flagellin detection during *Salmonella*-induced enterocolitis have not been fully elucidated.

Flagellar assembly is a complex process under tight regulation, with multiple signals that converge on the operon for the master regulator FlhDC, and multiple checkpoints that regulate transcription and translation of the structural machinery [41]. To understand the importance of flagellin recognition by the inflammasome *in vivo,* we used a *flgM* mutant [89]. The protein encoded by the gene *flgM* inhibits FliA, the sigma factor required for the transcription of class III flagellar genes [41]. *Salmonella* selectively represses flagellin expression in systemic sites during infection, suggesting that active evasion of immune surveillance is critical for *Salmonella* to establish systemic infection [50-52]. We and others have demonstrated previously that a functional FlgM is required to silence flagellin expression *in vivo*, and bacteria with deletions in *flgM* are attenuated during infection of mice in a flagellin-dependent manner [47,48,89]. FlgM-dependent flagellin silencing is critical for *Salmonella* to evade innate immune detection by the caspase-1 inflammasome, which is responsible for the attenuation of *flgM*$^{-}$ *Salmonella* [89]. Activation of the inflammasome by flagellin also limits intestinal inflammation during mucosal infection [89]. In this study, we used flgM and flagellin deficient *Salmonella* to help us dissect the function of each inflammasome component in flagellin detection in order to determine how these components modulate systemic spread of the bacterium and inflammation.

Our studies demonstrate that caspase-1 activation in vivo occurs primarily via Nlrc4. We dissected the role of Naip5 by performing oral and systemic infections. The role of Naip5 at systemic sites appears

to be necessary for all or most signaling via Nlrc4 and Caspase-1. Interestingly, mucosal infections (oral) revealed that Naip5 partially rescues the attenuated phenotype of flgM *Salmonella*, suggesting a very complex phenotype for Naip5 *in vivo*.

Methods

Ethics Statement. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All protocols were approved by the Institutional Animal Care and Use Committee of the University of Washington (protocol: 4031-01, Mucosal Immunity).

*Bacterial strains.*The experiments were performed using wildtype *Salmonella* enterica serovar Typhimurium strain SL1344 (WT) (from Brad Cookson, University of Washington). The flagellin-deficient (*fliC*$^-$/*fljB*$^-$) strain in this same background was also a gift from Brad Cookson (University of Washington). A *flgM* deletion mutant was made in SL1344 using lambda-red technology [77]. The deletion was confirmed by PCR, and also transferred to the *fliC*$^-$/*fljB*$^-$ mutant using P22 phage [78]. The deletion of *flgM* was once again confirmed by PCR. A constitutive GFP expressing plasmid (pDW5: ptetA::gfp; *gfp* downstream of *tetA* promoter in pBR322); reference PMID: 16803592) was introduced in *Salmonella* for fluorescent microscopy studies; the plasmid was a gift from Brad Cookson (University of

Washington). Bacteria were grown in Luria broth at 37°C with aeration.

Mouse infection. WT C57BL/6J (B6) mice were purchased from Jackson Labs and housed or bred in our facilities at the University of Washington. Caspase $1^{-/-}$ [79] and Toll-like receptor 5 deficient mice ($TLR5^{-/-}$) [70] on a B6 background were bred in our specific pathogen free (SPF) animal facilities. Animals were housed individually or in groups of up to five animals under standard barrier conditions in individually ventilated cages. 8-14 weeks old mice were used for infections throughout this study. One day before infection, food was withdrawn 4 h before oral gavage with 20 mg of streptomycin in 0.1 ml of PBS [54]. Afterwards, animals were supplied food ad libitum. At 20 h after streptomycin treatment, food was withdrawn again for 4 h before the mice were orally infected with 1000 CFU *S*. Typhimurium (delivered in 0.1 ml of PBS by gavage) or sterile PBS (control). The inoculum containing *Salmonella* was prepared by back-diluting an overnight culture 1:50 in LB + 50 µg/ml of streptomycin. After 4 hours, the concentration of bacteria was measured and diluted in cold PBS to a coentration of $1X10^4$ *CFU/ml*, and CFU of the inoculum was verified by plating on LB agar plates with 50 µg/ml streptomycin. Food was replaced immediately after gavage. Five days post-infection, mice were sacrificed by CO_2 asphyxiation, and blood and tissue (intestine, mesenteric lymph node, spleen, and liver) were promptly removed. Tissue samples from cecum and spleen were stored at -80 °C for RNA extraction. Bacterial burden was assessed by weighing and homogenizing the tissues in PBS with 0.025% Triton

X-100, and plating dilutions of the samples on MacConkey agar plates with streptomycin (50 ug/ml). For the homogenizing step, the ceca were scraped and blotted to remove fecal content.

Quantitative histologic assessment of inflammation in cecum, small intestine, liver, spleen and MLN. The formalin-fixed tissue was embedded in paraffin using standard protocols. 4 μm thick sections were stained with hematoxylin and eosin using standard procedures. A blinded pathologist (KDS) examined the slides and scored them according to the following criteria. Scores were assigned for changes to the cecum as follows: **submucosal expansion (S)** - 0 = no significant change, 1 = <25% of the wall, 2 = 25-50% of the wall, 3 =>50% of the wall; **mucosal neutrophilic infiltrate (M)** - 0 = no significant infiltrate, 1 = mild neutrophilic inflammation, 2 = moderate neutrophilic inflammation, 3 = severe neutrophilic inflammation; **lymphoplasmacytosis (L)** - 0 = no significant infiltrate, 1= focal infiltrates (mild), 2= multifocal infiltrates (moderate), 3 = extensive infiltrates involving mucosa and submucosa (severe); **goblet cells (G)** - 0 = >28/HPF, 1 = 11-28/HPF, 2 = 1-10/HPF, 3 = <1/HPF; **epithelial integrity (E)** - 0 = no significant change, 1 = desquamation (notable shedding of epithelial cells into the lumen), 2 = erosion (loss of epithelium with retention of architecture), 3 = ulceration (destruction of lamina propria).

Statistics. Significance was obtained by using the software GraphPad Prism (San Diego, CA). The Mann-Whitney test was used for all data where significance is shown. In all graphs, significance was

established and represented using the following system: * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$. One way ANOVA was used when comparing three groups or more, using either the Dunn's (non-Guassian) or Bonferroni's (Guassian) multiple comparisons test. Statistical significance was represented as above.

Results

***Salmonella typhimurium* requires *flgM* to evade inflammasome detection during intraperitoneal infection.** Flagellin can be detected by the Naip5-6/Nlrc4 inflammasome [27,62]. Because *Salmonella* downregulates flagellin expression *in vivo*, we tested whether or not the Naip5/Nlrc4 inflammasome contributes to control of intraperitoneal infection. We found no difference in the bacterial burden in the spleens or livers of WT mice infected with 100 CFU WT or flagellin-deficient *S*. Typhimurium (Fig. 1A,B), suggesting that *Salmonella* efficiently evades inflammasome-mediated detection of flagellin during intraperitoneal infection. *Salmonella* FlgM is an anti-sigma factor that binds FliA and prevents the expression of class III flagellar genes [41]. Upon completion of the flagellar basal body and hook, FlgM is secreted and FliA is released to activate class III promoters, resulting in completion of flagellar assembly [41]. Deletion of *flgM* results in constitutive activation of flagellar class III genes and disruption of autogenous regulation of flagellar assembly. FlgM mutants express more flagellin protein, have more flagella than WT *Salmonella*, and are attenuated in mice [47,48,89]. We recently demonstrated that *flgM*-deficient *Salmonella* are attenuated due to

Casp1-dependent innate immune detection [89]. During intraperitoneal flgM-deficient bacteria are significantly attenuated relative to both WT and flagellin-deficient *Salmonella* (Fig. 1 A, B), suggesting that evasion of flagellin detection is FlgM-dependent. Using *flgM*-deficient *Salmonella*, we determined that Casp1, Nlrc4 and Naip5 were all equally important for controlling intraperitoneal *Salmonella* infection (Fig. 1C,D). To confirm that *Salmonella* efficiently evade flagellin detection during intraperitoneal infection, we infected mice the knockout mice intraperitoneally with WT and flagellin-deficient *Salmonella*. Systemic infection of the liver and spleen by WT *Salmonella* was indistinguishable in WT, Nlrc4 and Naip5 mice (Fig. 1 E, F). Compared to WT mice Casp1$^{-/-}$ mice had a small but significant increase in CFU in the spleen but not liver (Fig. 1 E,F). Sytemic infection of the liver and spleen by flagellin-deficient *Salmonella* was indistinguishable in WT, Casp1$^{-/-}$ and Nlrc4$^{-/-}$ mice, indicating that these inflammasome components do not restrict flagellin-deficient *Salmonella* during intraperitoneal infection (Fig. 1G, H). Interestingly, Naip5$^{-/-}$ mice were protected from intraperitoneal infection by flagellin-deficient *Salmonella* and had decreased CFU in the spleen relative to WT and Casp1$^{-/-}$ mice, and in the liver relative to Casp1$^{-/-}$ mice (Fig 1G, H).

Differential requirement for Naip5/Nlrc4 inflammasome components during oral infection with WT *S*. Typhimurium. Because flagellin is expressed during the intestinal phase of *Salmonella* infection [50], we next investigated the role of the Naip5/Nlrc4 inflammasome during oral infection in streptomycin-

pretreated mice. Nlrc4$^{-/-}$ mice were highly susceptible to oral infection, and most of the mice (16/20) died by five days post-infection (Fig. 2A). Casp1$^{-/-}$ mice were slightly more susceptible than WT mice (Fig. 2A). In contrast, Naip5$^{-/-}$ mice were protected from oral *Salmonella* infection (Fig. 2A). We measured bacterial burden in tissues of mice at 5 days post-infection, and found that Nlrc4$^{-/-}$ and Casp1$^{-/-}$ mice had significantly more bacteria in the mesenteric lymph nodes, liver and spleen, compared to WT and Naip5$^{-/-}$ mice (Fig. 2B-E). At day 5 bacterial burden in tissues did not differ between WT and Naip5$^{-/-}$ mice (Fig. 2B-E). We also analyzed mice at 4 days post infection, when most of the Nlrc4$^{-/-}$ were still alive (18/20). At 4 days post-infection, Nlrc4 mice had significantly more bacteria in the mesenteric lymph nodes, spleen and liver compared to all other mice (Figure 3). Casp1$^{-/-}$ mice had more bacteria in the liver compared to WT mice, and Naip5$^{-/-}$ had fewer bacteria in the mesenteric lymph nodes, spleen and liver compared to all other mice (Figure 3).

Naip5 deficiency protects against aflagellate *Salmonella* infection. Intriguingly, Naip5$^{-/-}$ mice were protected against oral infection with WT *Salmonella* and Nlrc4$^{-/-}$ mice were more susceptible, suggesting that these molecules do not work in a simple linear pathway. During intraperitoneal infection with *flgM*-deficient *Salmonella*, Naip5 was protective and its function was concordant with Nlrc4 and Casp1. In contrast, during intraperitoneal infection with flagellin-deficient *Salmonella*, Naip5 promoted susceptibility. Similarly during oral infection with WT *Salmonella*, Naip5 promotes susceptibility, and its function was discordant with NlrC4 and Casp1.

This suggests that flagellin detection by Naip5 leads to Nlrc4- and Casp1-dependent protection against *Salmonella* infection, and that Naip5 also promotes *Salmonella* infection in a manner that is independent of Nlrc4, Casp1, and flagellin production by *Salmonella*. To test the hypothesis that decreased susceptibility to oral *Salmonella* infection seen in Naip5$^{-/-}$ mice does not require flagellin expression by *Salmonella*, we infected mice with flagellin-deficient *Salmonella*. WT, Casp1$^{-/-}$ and Nlrc4$^{-/-}$ mice were less susceptible to infection with flagellin-deficient *Salmonella*, and all mice survived to day 5 post-infection, suggesting that motility and/or flagellin expression are critical for the enhanced virulence of WT *Salmonella* in Nlrc4$^{-/-}$ and Casp1$^{-/-}$ mice. There was no difference in bacterial burden in the cecum between WT, Casp1$^{-/-}$, Nlrc4$^{-/-}$ and Naip5$^{-/-}$ mice; whereas Nlrc4$^{-/-}$ mice had significantly higher CFU in the spleen, liver and MLN, and Casp1$^{-/-}$ mice had significantly higher CFU in the liver (Fig. 4 B-D). Thus Nlrc4 and Casp1 protect against infection by aflagellate Salmonella, possibly through detection of Spi-1 type three secretory components, PrgI and PrgJ, via Naip1 and Naip2 [66,90]. Albeit the magnitude of protection is lower than that seen for WT *Salmonella*, suggesting that flagellin detection provides for the bulk of the Nlrc4 and Casp1 dependent protective response. In contrast Naip5$^{-/-}$ mice had less bacteria in the spleen and liver compared to the other mice, and in the MLN compared to Casp1$^{-/-}$ and Nlrc4$^{-/-}$ mice (Fig. 4B-D). Thus the paradoxical protection against oral *Salmonella* infection conferred by Naip5 deficiency was independent of flagellin expression by *Salmonella*.

The Naip5/Nlrc4 inflammasome protects against oral infection with *flgM*-deficient *Salmonella*. We orally infected streptomycin pretreated mice with 1000 CFU flgM-deficient *Salmonella*, and evaluated the mice after 5 days. Both Nlrc4$^{-/-}$ and Casp1$^{-/-}$ mice were equivalent to each other, and had more bacteria in all organs compared to WT and Naip5$^{-/-}$ mice (Fig. 5A-D). The Naip5$^{-/-}$ mice had an intermediate phenotype, and had more bacteria in the spleen, liver and mesenteric lymph nodes than WT mice, but less bacteria in these organs than Nlrc4$^{-/-}$ and Casp1$^{-/-}$ mice (Fig. 5B-C). Both WT and Naip5 mice had similar counts of bacteria in the cecum, and both had significantly less cecal CFU than Casp1$^{-/-}$ and Nlrc4$^{-/-}$ mice (Fig. 5A). Thus Naip5 protects against infection with flgM-deficient bacteria, but the intermediate phenotype suggests that this protective effect is counterbalanced by the flagellin-, Nlrc4- and Casp1-independent activity that promotes infection.

Asc provides partial protection during oral *Salmonella* infection. Asc is required for efficient IL-1B processing by the Naip5/Nlrc4 inflammasome [91]. Asc is also a component of the Nlrp3 inflammasome, which has been implicated in protection against oral *Salmonella* infection [91]. To determine if Asc and Nlrp3 contribute to control of oral *Salmonella* infection, we examined WT, Asc$^{-/-}$ and Nlrp3-/- mice. At 5 days post oral infection with either WT or flgM-deficient *Salmonella,* Nlrp3$^{-/-}$ mice were indistinguishable from WT mice (Fig. 6A-H). In contrast, Asc$^{-/-}$ had significantly more bacteria than WT mice in the spleen and liver, when infected with WT or flgM-deficient bacteria, and in the mesenteric lymph nodes when infected

with WT Salmonella (Fig. 6A-H). The Nlrp3$^{-/-}$ mice had more bacteria in the liver compared to WT mice when infected with flgM-deficient *Salmonella* (Fig. 6G), suggesting that Nlrp3 may play a modest, non-redundant role in *Salmonella* infection.

Discussion

In a previous study we demonstrated that proper regulation of flagellin production by FlgM is necessary for *Salmonella*'s virulence [89]. In this study, we expanded our model to incorporate the role of other inflammasome components in the detection of flagellin *in vivo* and we defined the role of each inflammasome component during oral and systemic (intraperitoneal) routes of infection. We previously showed that flagellin is a potent activator of the innate immune system during oral infection with *flgM*-deficient *Salmonella*, leading to substantial protection, up to 4 logs less bacterial burden in tissues and elimination of the intracellular lifestyle of *Salmonella* [89]. Herein we showed that during intraperitoneal infection flagellin detection is largely evaded by WT *Salmonella*. Based on our studies with flgM-deficient *Salmonella* flagellin detection during systemic infection relies predominantly on Naip5, Nlrc4 and Caspase-1. Interestingly, intraperitoneal infection with flagellin-deficient *Salmonella* revealed that Naip5 paradoxically promotes *Salmonella* infection in a flagellin independent manner. This activity appears to be independent of Casp1, although Nlrc4 may have a partial role since the Nlrc4$^{-/-}$ mice could not be distinguished from WT, Casp1$^{-/-}$ or Naip5$^{-/-}$ mice (Fig. 1 G,H). Because the Casp1$^{-/-}$ mice also harbor the natural mutation in

Casp11, it is possible that the $Casp1^{-/-}$ phenotype reflects a combination of defective Casp1 and Casp11. Future studies using individual Casp1 and Casp11 deficient mice should help sort out this issue.

importance of Naip5 diminishes in orogastric flgM infections suggesting that it possesses positive and negative aspects in a *Salmonella* infection. While in flagellin deficient and WT *Salmonella* infections, Naip5 deficiency is protective. Under circumstances where flagellin is being produced by *Salmonella*, as is the case in flgM oral infections, Naip5 interaction with *Salmonella*'s flagellin has detrimental effects and leads to higher bacterial burden than those observed in WT mice.

In this report, we show that the Naip5/Nlrc4/Caspase-1 inflammasome pathway recognizes *Salmonella*'s flagellin *in vivo*. Our results indicate that Nlrc4 is required for mucosal and systemic recognition of *Salmonella'*s flagellin. The bacterial burden in $Nlrc4^{-/-}$ and $Casp1^{-/-}$ mice infected with flagellin- or *flgM*-deficient *Salmonella* were largely indistinguishable suggesting that caspase-1 activation by flagellin occurs mainly via Nlrc4. Interestingly, in a WT *Salmonella* infection, the survival rate of $Nlrc4^{-/-}$ mice was reduced compared to $Casp1^{-/-}$ mice and the bacterial burden in the liver of $Nlrc4^{-/-}$ mice was significantly higher at day 4 post-infection (Fig. 3). Collectively, these data suggests that $Nlrc4^{-/-}$ mice are more susceptible to infection than $Casp1^{-/-}$ mice. Recently, it was discovered that $Casp1^{-/-}$ mice also harbor an inactivating mutation in caspase-11 that is present in 129

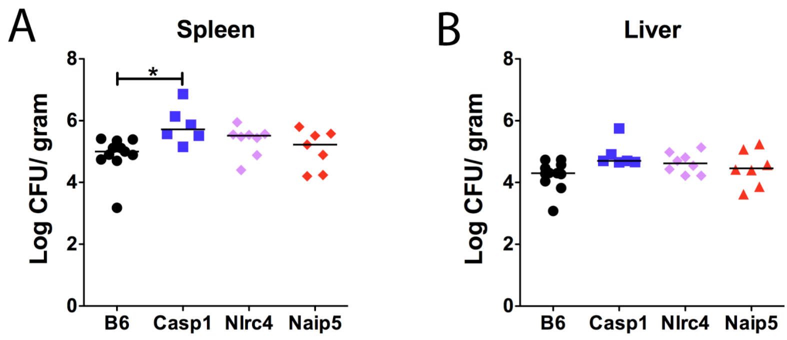
strains of mice, the source of the embryonic stem cells used to make the Casp1$^{-/-}$ mice. [92]. Caspase-11 deficient mice are protected against a wide range of intracellular pathogens [93,94]. Thus Nlrc4$^{-/-}$ mice increased susceptibility to infection compared to casp1$^{-/-}$ mice could be attributed to 1) lack of caspase-1 activation, and 2) functional caspase-11.

The contribution of Nlrc4 in a *Salmonella* infection has been studied in detail with conflicting results. Our results agree with a previous observation by the Aderem group demonstrating the importance of Nlrc4 in caspase-1 recognition of *Salmonella*'s flagellin [95]. The Gewirtz lab also determined that Nlrc4 is important in survival and controlling bacterial burden in mice [96]. Although work by the Nunez group observed a significant increase in susceptibility in Nlrc4$^{-/-}$ mice in an oral *Salmonella* infection, this observation was only prevalent in the Balb/c but not the C57BL/6 background [31]. The Monack lab also studied the role of Nlrc4 in an oral infection and did not observe an effect in bacterial burden in Nlrc4$^{-/-}$ mice infected with *Salmonella* compared to WT mice [29].

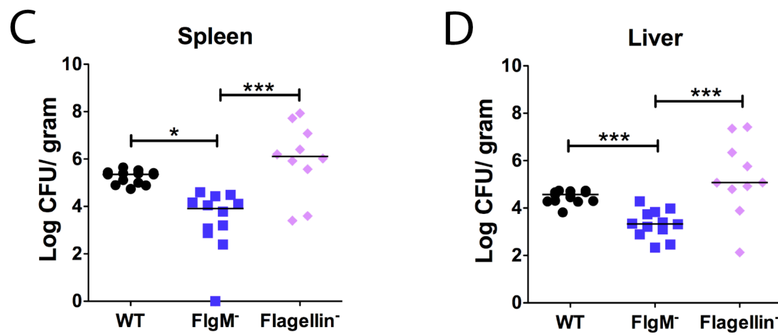
In a WT Salmonella infection, flagellin is strongly regulated in order to avoid inflammasome dependent immune recognition as demonstrated in this study (Fig 1A, B, C, D and Fig 2), but also by the Cookson and the Gewirtz groups [50,51,89]. However, the role of Nlrp3 and ASC *in vivo* has not been extensively studied. ASC is important for IL-1B processing while Nlrp3 is believed to activate the inflammasome by an unknown ligand, indpendent of flagellin or a

T3SS [29]. Our studies demonstrate that Nlrp3 is dispensable for protection against WT or flgM *Salmonella*. However, ASC is modestly important in preventing systemic spread by either WT of flgM *Salmonella.*

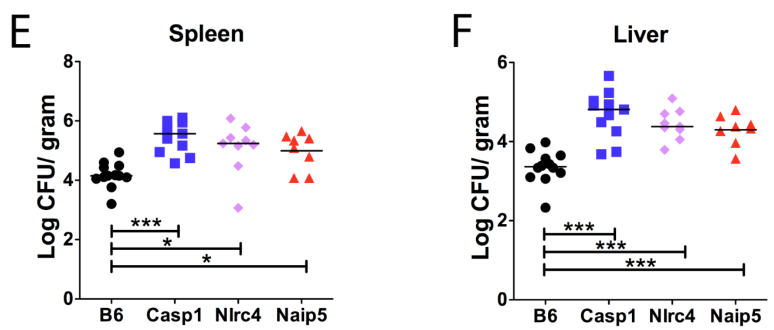
WT Salmonella infection:



C57BL/6 WT mice:



flgM deficient Salmonella infection:



Flagellin deficient Salmonella infection:

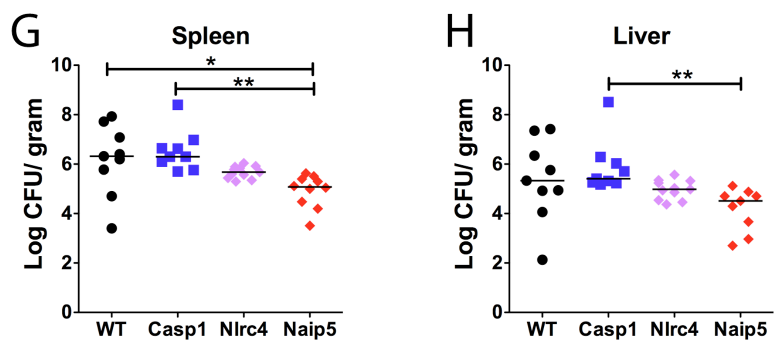
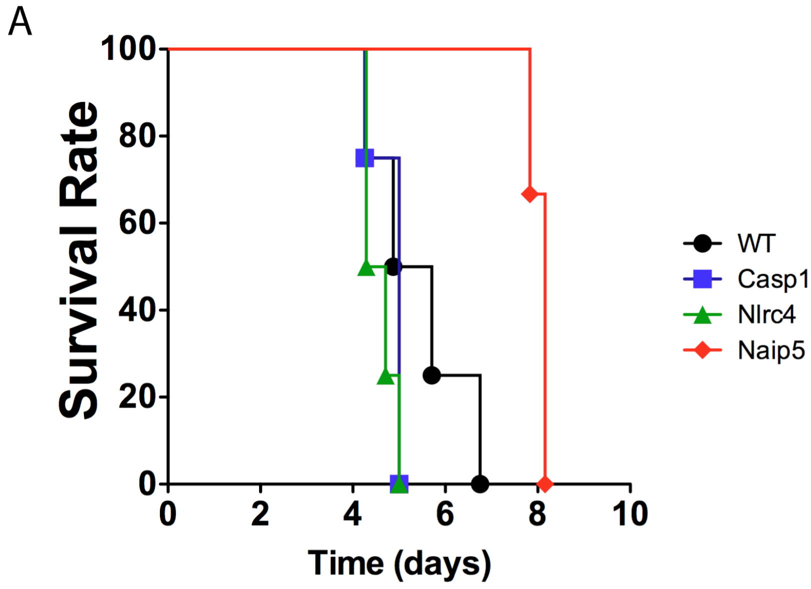
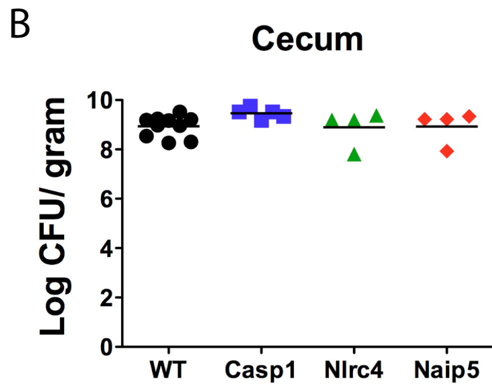


Figure 1. Salmonella efficiently evades the inflammasome in systemic infection. C57BL/6 WT (n=11), casp1-/- (n=6), Nlrc4-/- (n=8) and Naip5-/- (n=7) mice infected with 100 CFU of WT Salmonella. Spleen (A) and liver (B) bacterial burden was recorded 3 days after intraperitoneal infection. Bacterial burden in the spleen (C) and liver (D) of WT C57BL/6 mice infected with WT (n=9), FlgM (n=12) and flagellin deficient (n=10) Salmonella was obtained 3 days after intraperitoneal infection. WT (n=12), casp1-/- (n=11), Nlrc4-/- (n=10) and Naip5-/- (n=8) mice were infected intraperitoneally with flgM deficient Salmonella, bacterial burden was assessed three days after infection in the spleen (E) and liver (F). Bacterial burden was determined after three days of intraperitoneal infection with flagellin deficient Salmonella in the spleen (G) and liver (H) in WT (n=9), casp1-/- (n=9), Nlrc4-/- (n=11) and Naip5-/- (n=10). All figures represent the combined data of two independent experiments. Mann-Whitney test *= p 0.05, **= p 0.01, ***= p 0.001.

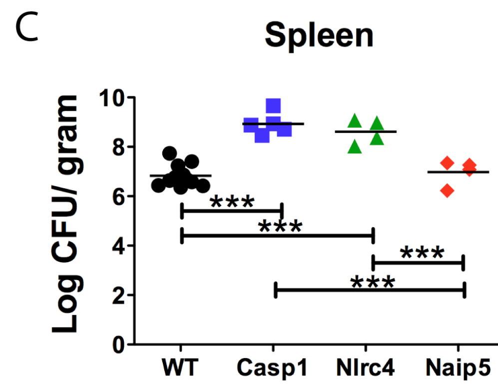
A



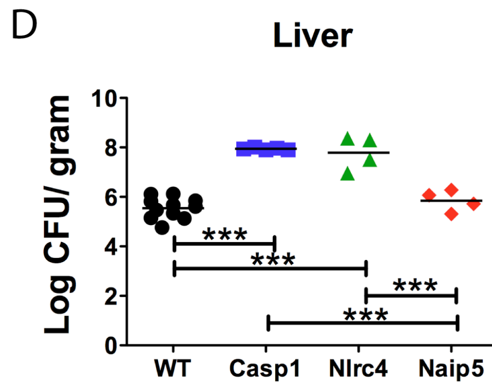
B



C



D



E

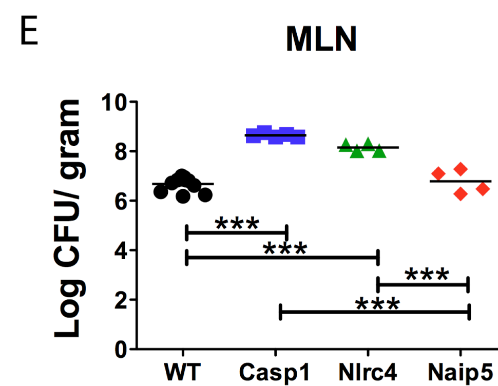


Figure 2. Flagellin detection by the inflammasome influences mucosal infection by Salmonella. Survival rate graph (A) showing WT (n=7), casp1-/- (n=9), Nlrc4-/- (n=7) and Naip5-/- (n=7) mice infected with 100 cfu WT Salmonella orally. WT (n=11), casp1-/- (n=5), Nlrc4-/- (n=4) and Naip5-/- (n=4) mice infected orally with 1000 cfu WT Salmonella for 5 days. Graphs depict bacterial burden in the cecum (B), spleen (C), liver (D) and MLN (E). Figure A represents the combined data of two independent experiments. Figure B, C, D, E represent the combineddata of two independent experiments. Mann-Whitney test *= p 0.05, **= p 0.01, ***= p 0.001.

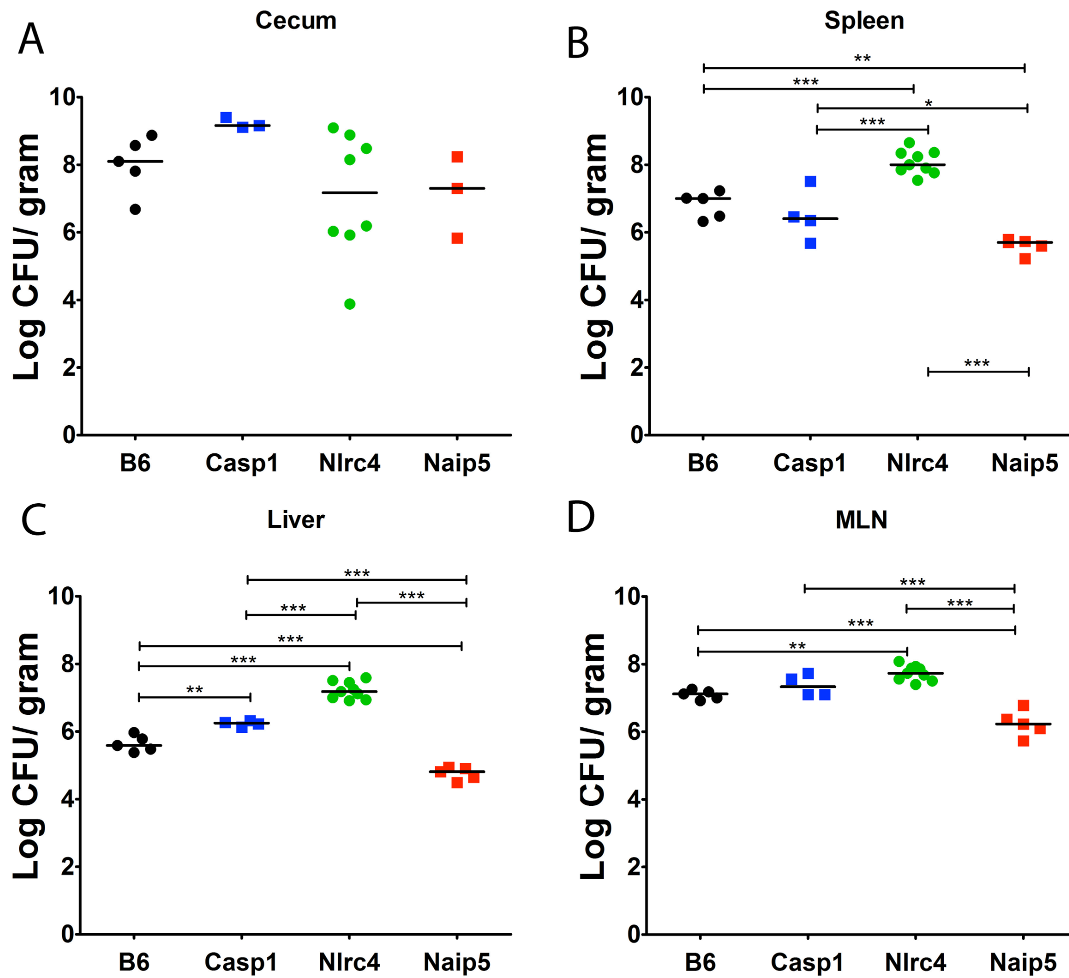


Figure 3. Nlrc4 deficiency increases susceptibility to Salmonella infection. WT (n=5), casp1-/- (n=4), Nlrc4-/- (n=9) and Naip5 (n=4) mice infected orally with 1000 cfu of WT Salmonella for 4 days. Graphs depict bacterial burden in cecum (A), spleen (B), liver (C) and MLN (D). Figures A-D represent one experiment. Mann-Whitney test *= p 0.05, **= p 0.01, ***= p 0.001.

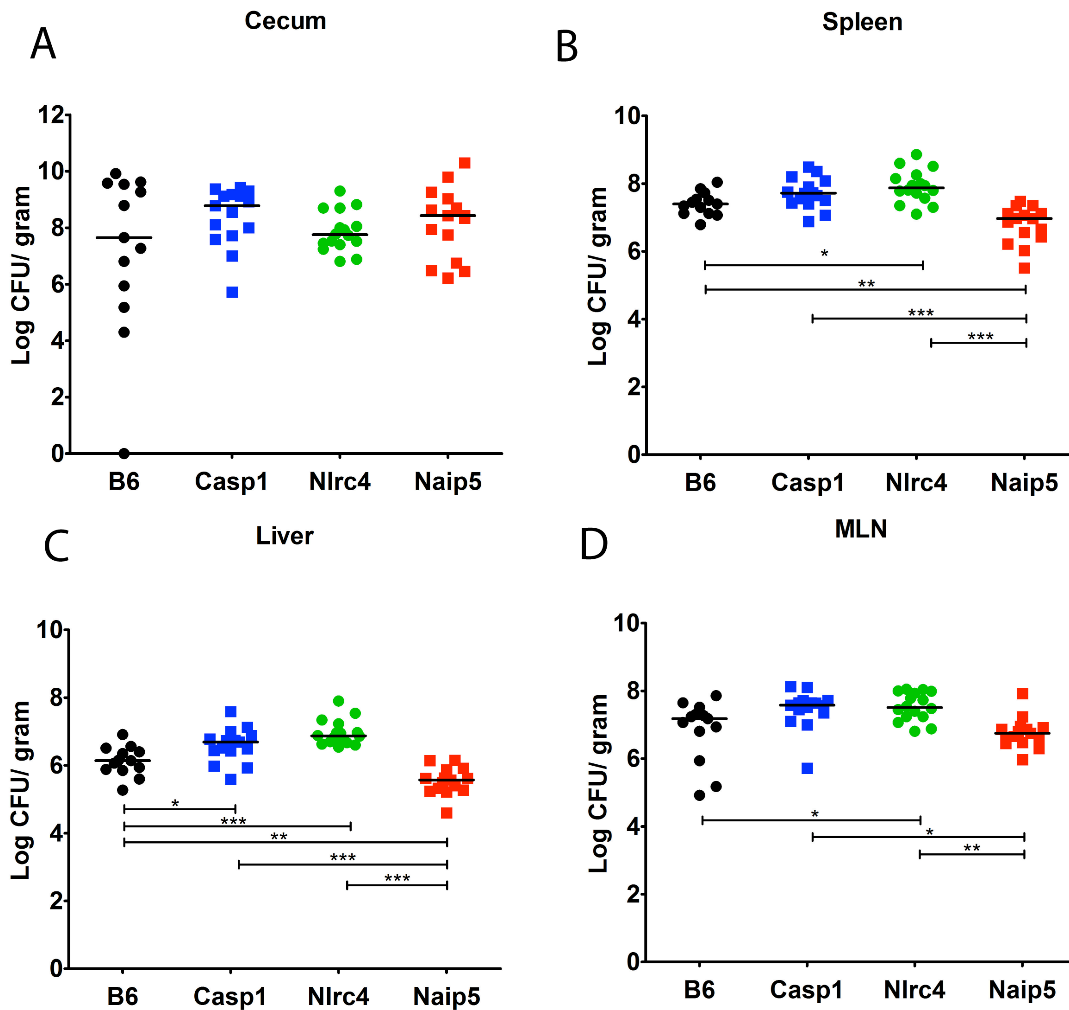


Figure 4. Naip5 deletion protects against flagellin deficient Salmonella. WT (n= 13), casp1-/- (n=15), Nlrc4-/- (n=16) and Naip5-/- (n=15) mice infected orally with 1000 cfu flagellin deficient Salmonella for 5 days, cecum (A), spleen (B), liver (C), MLN (D). Figures A-D represent the combined data of three independent experiments. Mann-Whitney test *= p 0.05, **= p 0.01, ***= p 0.001.

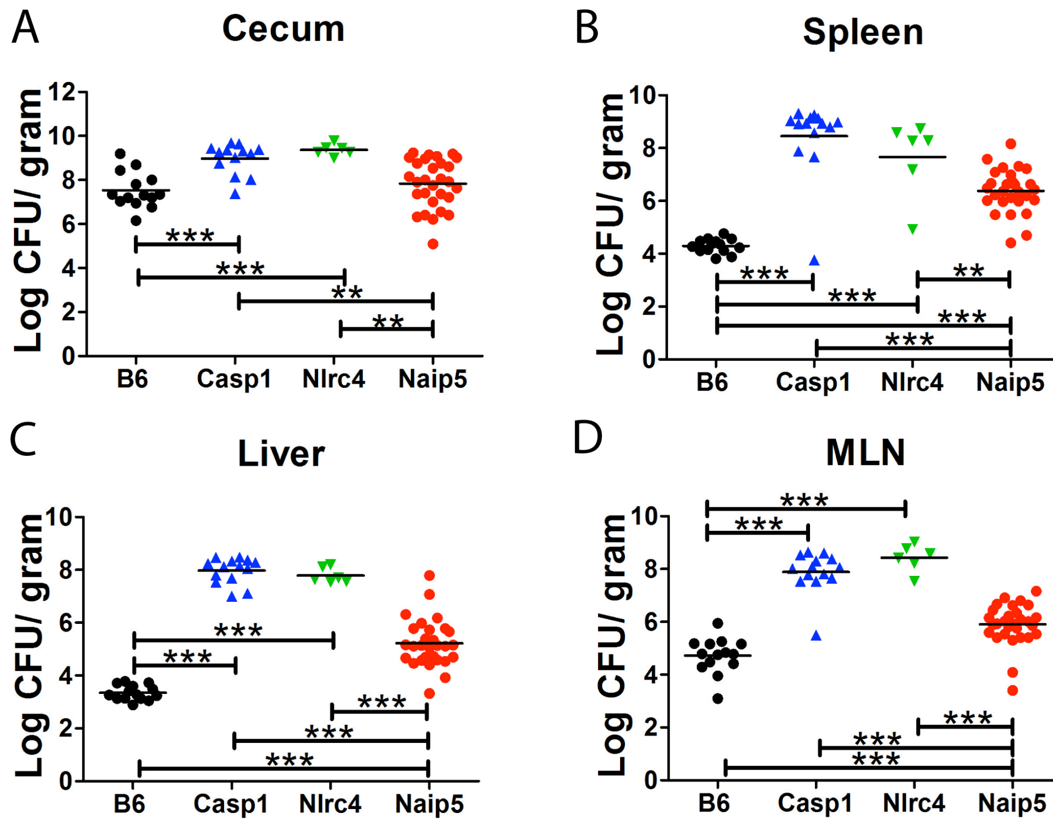
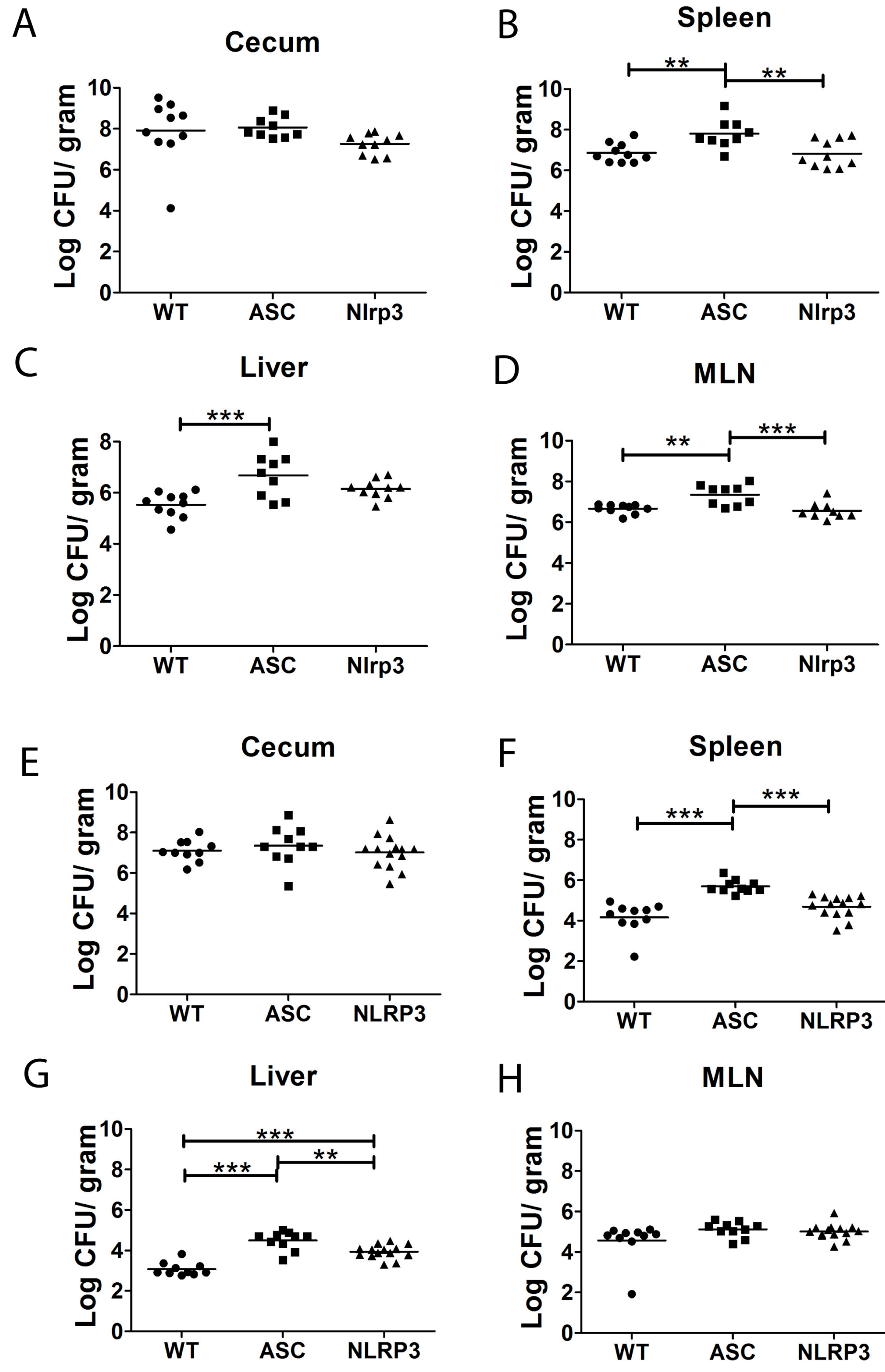


Figure 5. The Naip5/Nlrc4 inflammasome protects against oral flgM Salmonella infection. WT (n=14), casp1-/- (n=14), Nlrc4-/- (n=6), Naip5-/- (n=29) mice infected orally with 1000 cfu flgM Salmonella for 5 days, cecum (A), spleen (B), liver (C) and MLN (D). Figures A-D represent the combined data of three independent experiments. Mann-Whitney test *= p 0.05, **= p 0.01, ***= p 0.001.


A
Cecum
Log CFU/ gram
10
8
6
4
2
0
WT
ASC
Nlrp3
B
Spleen
**
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Log CFU/ gram
10
8
6
4
2
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WT
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Nlrp3
C
Liver
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MLN
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WT
ASC
NLRP3

Figure 6. ASC provides partial protection against oral Salmonella infection. WT (n=10), ASC-/- (n=9) and Nlrp3 (n=10) mice infected orally with 1000 cfu WT Salmonella for 5 days, cecum (A), spleen (B), liver (C), MLN (D). WT (n=10), ASC-/- (n=10) and Nlrp3 (n=14) mice were orally infected with 1000 cfu flgM Salmonella for 5 days, cecum (E), spleen (F), liver (G) and MLN (H). Figures (A-H) represent the combined data of two independent experiments. Mann-Whitney test *= p 0.05, **= p 0.01, ***= p 0.001.

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Chapter 4: Conclusions

In this dissertation, we have analyzed the role of *Salmonella*'s flagellin recognition in an *in vivo* mouse model. This is one the most detailed analysis of the interaction of flagellin with the innate host system to date *in vivo*. This study is also the first one to study the roles both TLR5 and caspase-1 dependent flagellin recognition pathways simultaneously, allowing us to make a direct comparison on the importance and contribution that each one plays in innate immunity. Our studies indicate that *Salmonella* tightly regulates flagellin production in order to evade innate immune detection in the host, and that flagellin dysregulation has detrimental effects on *Salmonella*'s virulence and fitness as a pathogen. A lab generated Salmonella mutant that is deficient in the *flgM* gene overproduces flagellin and it is highly attenuated *in vivo*. The attenuated phenotype observed in *flgM Salmonella* is dependent on flagellin, as deletion of flagellin restores virulence. Flagellin recognition by the host also protects the mucosal surfaces and results in decreased inflammation in the cecum as observed in our histological analysis of infected mice.

Flagellin recognition relies on two independent pathways dependent on either i) TLR5   ii) Naip5/Naip6/Nlrc4/Caspase-1. Our studies used the flagellin overproducing Salmonella (*flgM*) as a tool to study the role of each known flagellin recognition pathways *in vivo* in a mouse infection model. Paradoxically TLR5 increases susceptibility to a *Salmonella* infection. Mice deficient in TLR5 were protected from

infection and had lower bacterial burden in the cecum and higher inflammatory scores suggesting that TLR5 has an important role in determining the inflammatory response of the gut. We believe that this could be attributed to alterations in the commensal population that inhabits the TLR5- gut, as shown by the Gerwitz group [71,72]. On the other hand, caspase-1 protects against *Salmonella* infection, systemic dissemination and inflammation. Caspase-1 is required for pyroptosis and the cleavage of cytokines into their mature forms. We believe that pyroptosis is responsible for flagellin recognition and the subsequent bacterial clearance and mucosal protection. Immunofluorescence imaging showed that caspase-1 deficient macrophages have significantly higher levels of bacteria suggesting that caspase-1 dependent cell death prevents *Salmonella* from growing within host cells.

The caspase-1 inflammasome relies on Naip5, Naip6 and Nlrc4 for flagellin recognition. We believed that the inflammasome components, Naip5, Nlrc4 and Caspase-1, have distinct roles at systemic and mucosal sites. In order to dissect the importance of the flagellin dependent inflammasome, we infected mice deficient in each of these inflammasome components with WT, flgM and flagellin deficient systemically (intraperitoneal injection) or orally (orogastric delivery). In our studies, we demonstrated that each component of this inflammasome plays a role in flagellin recognition *in vivo* both at the mucosal sites and systemically. Oral infection with WT *Salmonella* demonstrated that Nlrc4 and caspase-1 are both equally important in flagellin recognition, bacterial clearance and prevention of systemic spread. However, Naip5 proved to be dispensable in

protecting against bacterial burden in all organs examined, suggesting that Naip5 contribution to caspase-1 activation is unnecessary in a wildtype *Salmonella* infection. Interestingly, survival studies showed that Nlrc4 deficient mice succumb rapidly to infection compared to caspase-1 deficient mice. Based on recent studies by Petr Broz in Denise Monack's group and Youssef Aachoui in Ed Miao's group, Caspase-11 deficient mice are protected against intracellular bacteria [93,94,97]. The caspase-1 deficient mice used in these studies are both deficient in caspase-1 and caspase-11. We believe that Nlrc4 increased susceptibility to *Salmonella* compared to caspase-1 deficient mice is due to two distinct processes. On one hand, Nlrc4 deficient mice are not able to activate caspase-1, but also caspase-11 remains active and it increases susceptibility to infection as studied by the Monack and Miao groups [93,94,97].

Orogastric *flgM* attenuation was dependent on Nlrc4, but partially dependent on Naip5, suggesting a role for Naip6. Systemic infections using flgM demonstrated that most of the caspase-1 dependent protection occurred via Naip5. These data strongly suggest that Naip5 has protective effects only when the pathogen produces flagellin *in vivo* as is the case of the flgM mutant. However, in flagellin deficient Salmonella infection, Naip5 deficient mice were protected and experienced lower bacterial burden even compared to WT mice. In addition, survival experiments demonstrated that Naip5 deficient mice lived longer, up to 3 more days than WT mice. These data is interesting and enigmatic as it suggests that a functional Naip5 is detrimental to the innate immune's ability to fight infection. The

phenotype could be explained if Naip5 deficient mice have a different microbiota that prevents Salmonella colonization. However, studies on the gut microbiota of Naip5 deficient mice have not been done and we can only speculate on the rational behind these paradoxical results.

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