THE INFLAMMATORY CASPASES COORDINATE MUCOSAL RESTRICTION OF SALMONELLA ENTERICA SEROVAR TYPHIMURIUM

by

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The inflammatory caspases coordinate mucosal restriction of *Salmonella enterica* serovar Typhimurium

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Abstract

We investigated the role of the inflammatory caspases during Salmonella enterica serovar Typhimurium infection of murine intestinal epithelial cells (IECs). Streptomycin-pretreated wildtype C57BL/6, Casp1/11 deficient (-/-), Casp1-/- and Casp11-/- mice were orally infected and S. Typhimurium burdens determined at 18h-7d post infection (p.i.). Increased cecal and luminal pathogen burdens were observed for all caspase-deficient mice as compared to wildtype, which correlated with increased intracellular S. Typhimurium loads in the crypt IECs. Interestingly, cecal pathology scores for all inflammatory caspase mice were decreased compared to wildtype mice, especially with regard to 'epithelial integrity' and 'goblet cell loss'. To determine if the increased intracellular pathogen burdens were due to the loss of IEC-intrinsic inflammasomes, cell lines and enteroid monolayers derived from each genotype and infected. These studies revealed significantly increased intracellular burdens in caspase-deficient monolayers in concert with a marked decrease in IEC sloughing and cell death. In human epithelial monolayers, siRNA-depletion of caspase-4, a human ortholog of caspase-11, led to increased bacterial colonization as well as increased secretion of the proinflammatory cytokine, interleukin (IL)-18. Inflammatory caspase activity was measured in enteroid monolayers and peak activity in wildtype cells correlated with shedding, suggesting IEC-intrinsic inflammasomebased restriction of S. Typhimurium occurs through infected IEC expulsion. To examine the effect of inflammasome signaling on overall mucosal defense, mucus layer thickness was evaluated by immunofluorescence staining. At 18h p.i., wildtype tissues demonstrated a dramatic increase in mucus thickness while only a marginal increase was observed in caspase deficient mice. Also, expression of the antimicrobial lectins Reg3 γ and β were attenuated in all caspasedeficient mice. Mucin release and Reg3 γ and β induction has been previously linked to the

cytokine IL-22. We detected higher IL-22 levels in infected wildtype mice and when IL-22 was neutralized, wildtype mice carried increased S. Typhimurium burdens and decreased infection-induced mucin secretion and Reg3 γ and β induction. No differences were observed in Casp1/11–/– mice treated with neutralizing antibody or isotype control. These results thus indicate that the intestinal mucosa utilizes inflammasome signaling to coordinate multiple layers of innate defense at the gut surface to ultimately restrict enteric pathogen infections and systemic spread.

Lay Summary

Intestinal epithelial cells (IECs) are located at the interface between the gut lumen and the underlying mucosal immune system. Here, they play a central role in the coordination of intestinal homeostasis, tempering pro-inflammatory responses against the intestinal microbiota, while remaining vigilant and rapidly responsive when exposed to a noxious stimulus such as an enteric pathogen. One early response mechanism by which IECs engage in immune defense is through the activation of the inflammasome which mobilizes the inflammatory caspases; Caspase-1 and -11. Here, we investigate the role of the inflammasome in IEC as well as overall intestinal mucosal defense against the enteric pathogen *Salmonella enterica* serovar Typhimurium.

Preface

All of the work presented hereafter was conducted in the laboratory of Dr. Bruce A. Vallance at the University of British Columbia, BC Children's Hospital Research Institute. All projects and associated methods were approved by the University of British Columbia's Animal Care Committee [certificate #A14-0253 and #A15-0211].

A version of Chapter 1 has been published [Crowley SM, Knodler LA and Vallance BA. 2016. *Salmonella* and the Inflammasome: Battle for Intracellular Dominance. *Curr Top Microbiol Immunol.* 2016;397:43-67; Crowley SM, Vallance BA and Knodler LA. 2017. Noncanonical inflammasomes: Antimicrobial defense that does not play by the rules. *Cell Microbiol.* 2017 Apr;19(4); Allaire JM[†], Crowley SM[†], Law HT, Chang SY, Ko HJ and Vallance BA ([†] - joint first authors). 2018. The Intestinal Epithelium: Central Coordinator of Mucosal Immunity. *Trends Immunol.* 2018 Sep;39(9):677-696]. Text and figures are used with permission from applicable sources.

A version of Chapter 2 has been published in Cell, Host & Microbe [Knodler LA, Crowley SM, Sham HP, Yang H, Wrande M, Ma C, Ernst RK, Steele-Mortimer O, Celli J and Vallance BA. 2014. Noncanonical inflammasome activation of caspase-4/caspase-11 mediates epithelial defenses against enteric bacterial pathogens. *Cell Host Microbe* 13;16(2):249-256]. Text and figures are used with permission from the journal. I was a co-author, responsible for all mouse infections (oral and *i.p.*), immunostaining and Western blots as well as the manuscript composition and figures relating to these experiments. I am responsible for the data presented in Figure 2.5 (Western blot), Figure 2.7 and Figure 2.8. Ho Pan Sham was involved in the initial

murine infections, Hyungjun Yang conducted murine ELISA and Caixia Ma provided assistance with animal experimentation. Leigh A Knodler was the lead author and responsible for experimental design, all *in vitro* experiments, data analysis and manuscript composition. Marie Wrande conducted RNA extraction and qPCR, Robert K Ernst provided purified *Salmonella* LPS, Olivia Steele-Mortimer provided experimental funding and Jean Celli performed the digitonin permeabilization as well as experimental funding. Bruce A Vallance was the supervisory author and was involved throughout the project in study design and manuscript edits.

A version of Chapter 3 has been accepted in PLOS Pathogens [Crowley SM, Han X, Allaire JM, Stahl M, Rauch I, Knodler LA and Vallance BA. 2020. Intestinal restriction of *Salmonella* Typhimurium requires caspase-1 and caspase-11 epithelial intrinsic inflammasomes. *Submitted*.]. I was the lead investigator, responsible for all major areas of study design, data collection and analysis, as well as manuscript composition. Xiao Han and Joannie M. Allaire assisted in enteroid monolayer infections and aided in data analysis. Martin Stahl prepared media for enteroid growth as well as optimized enteroid methods. Isabella Rauch provided $Casp1^{-/-}$ mice. Bruce A. Vallance was the supervisory author and alongside Leigh A. Knodler was involved throughout the project in study design, data interpretation, writing the discussion and manuscript edits.

I was the lead investigator for Chapter 4 where I was responsible for all major areas of study design, data collection and analysis, as well as manuscript composition. Joannie M. Allaire, Franziska Gräf and Xiao Han assisted in histopathological scoring. Isabella Rauch provided $Casp1^{-/-}$ mice. Bruce A. Vallance was the supervisory author and alongside Leigh A. Knodler

was involved throughout the project in study design, result discussion and manuscript edits. A version of this chapter will be submitted for publication.

Finally, Chapter 5 includes an adapted figure (Figure 5.1) from our review manuscript [Allaire JM[†], Crowley SM[†], Law HT, Chang SY, Ko HJ and Vallance BA ([†] - joint first authors). 2018. The Intestinal Epithelium: Central Coordinator of Mucosal Immunity. *Trends Immunol*. 2018 Sep;39(9):677-696] used with permission from the journal.

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List of Symbols and Abbreviations

| α | Alpha |
|-----------|---|
| β | Beta |
| γ | Gamma |
| μ | Micro |
| < | Less than |
| > | Greater than |
| 0 | Degree |
| ± | Plus-minus |
| % | Percent |
| _/_ | Deficient |
| 3D | Three-dimensional |
| AIM2 | Absent in melanoma 2 |
| ASC | Apoptosis-associated speck-like protein |
| ATCC | American Type Culture Collection |
| BCCHRI | BC Children's Hospital Research Institute |
| BIR | Baculovirus inhibitor of apoptosis protein repeat |
| BMDM | Bone marrow derived macrophage |
| BP | Binding protein |
| С | Celsius |
| CARD | Caspase activation and recruitment domain |
| Casp/CASP | Caspase (lower case – murine; upper case – human) CFU |
| CCAC | Canadian Council on Animal Care |

| CFU | Colony forming unit |
|-------|---|
| CK19 | Cytokeratin 19 |
| Ctrl | Control |
| DAMPs | Danger-associated molecular patterns |
| DAPI | 4',6-diamidino-2-phenylindole |
| DC | Dendritic cell |
| DMEM | Dulbecco's Modified Eagle Medium |
| DNA | Deoxyribonucleic acid |
| DSS | Dextran sulphate sodium |
| E.g. | Example |
| ELISA | Enzyme-linked immunosorbent assay |
| EpCAM | Epithelial cell-adhesion molecule |
| EPEC | Enteropathogenic E. coli |
| FAE | Follicle-associated epithelium |
| FBS | Fetal bovine serum |
| FIIND | Function to find domain |
| GAPs | Goblet cell-associated antigen passages |
| GBP | Guanylate-binding protein |
| GFP | Green fluorescent protein |
| GI | Gastrointestinal |
| Gsdmd | Gasdermin D |
| h | Hour |
| H&E | Hematoxylin and eosin |

| HBSS | Hank's balanced salt solution |
|--------|---|
| HIN200 | Hemopoietic expression, interferon-inducibility, nuclear localization |
| hNAIP | Human NAIP |
| i.e. | id est "in other words" |
| IEC | Intestinal epithelial cell |
| IEL | Intraepithelial lymphocyte |
| IFN | Interferon |
| IgG | Isotype control |
| IL | Interleukin |
| αIL-22 | IL-22 neutralizing antibody |
| ILC | Innate lymphoid cell |
| i.p. | Intraperitoneal |
| IRF3 | Interferon regulatory factor 3 |
| IVOC | Human intestinal in vitro organ culture |
| L | Liter |
| LB | Lysogeny broth |
| LDH | Lactate dehydrogenase |
| LPS | Lipopolysaccharide |
| L.u. | Lumen |
| LRR | Leucine rich repeat |
| m | Milli |
| m | Meter |
| M1 | Classically activated macrophage |

| M2 | Alternatively activated macrophage | |
|--|---|--|
| MALDI-TOF | Matrix-assisted laser desorption/ionization-time of flight | |
| МАРК | Mitogen-activated protein kinase | |
| mEGF | Murine epidermal growth factor | |
| MLN | Mesenteric lymph node | |
| MOI | Multiplicity of infection | |
| mROS | Mitochondrial reactive oxygen species | |
| mtDNA | Mitochondrial DNA | |
| NACHT | NAIP, C2TA (MHC class 2 transcription activator), HET-E | |
| (incompatibility locus protein from Podospora anserina) and TP1 (telomerase-associated | | |
| protein) | | |
| Naip1-6 | Neutronal apoptosis inhibitor protein 1-6 | |
| NF-κB | Nuclear factor kappa-light-chain-enhancer of activated B cells | |
| NK | Natural Killer | |
| NLRs | NOD-like receptors | |
| Nlrc4 | NOD-like receptor family, caspase-activating and recruitment domain | |
| containing protein 4 | | |
| Nlrp3 | NOD-like receptor family, pyrin domain containing 3 | |
| Nlrp1b | NOD-like receptor family, pyrin domain containing 1 | |
| Nlrp6 | NOD-like receptor family, pyrin domain containing 6 | |
| NOD | Nucleotide-binding oligomerization domain | |
| NRT | No reverse transcriptase | |
| NT | Non-targeting control | |

| n.s. | Not significant |
|-----------|--|
| PAMPs | Pathogen-associated molecular patterns |
| PBS | Phosphate-buffered saline |
| PCR | Polymerase chain reaction |
| Pen Strep | Penicillin streptomycin |
| p.i. | Post infection |
| PI | Propidium iodide |
| PITs | Pore-induced intracellular traps |
| PFA | Paraformaldehyde |
| PMN | Polymorphonuclear leukocytes |
| PRR | Pattern recognition receptors |
| PVDF | Polyvinylidene fluoride |
| PYD | Pyrin |
| qPCR | Quantitative polymerase chain reaction |
| RFP | Red fluorescent protein |
| RIPA | Radioimmunoprecipitation assay buffer |
| RNA | Ribonucleic acid |
| ROS | Reactive oxygen species |
| SCV | Salmonella containing vacuole |
| SD | Standard deviation |
| SDS | Sodium dodecyl sulfate |
| SEM | Standard Error |
| siRNA | Small interfering RNA |

| SN | Supernatants |
|----------------|--|
| SPI | Salmonella pathogenicity island |
| STAT6 | Signal transducer and activator of transcription 6 |
| Strep | Streptomycin |
| S. Typhimurium | Salmonella enterica serovar Typhimurium |
| T3SS | Type three secretion system |
| TCA | Tricarboxylic acid cycle |
| TLRs | Toll-like receptors |
| TNF-α | Tumor necrosis factor alpha |
| UEA-1 | Ulex europaeus agglutinin I |
| WCL | Whole cell lysates |
| Wnt3a | Wnt Family Member 3A |
| WRN | Wnt3a/R-spondin/Noggin secretion media |
| ZO-1 | Zonula occludens-1/Tight junction protein-1 |

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Chapter 1: Introduction

1.1 Salmonella

Salmonella is a genus of pathogenic, motile, Gram-negative bacteria that are part of the Enterobacteriaceae family. It is divided into two species: Salmonella bongori and Salmonella enterica, which both cause enteric disease in a wide range of animals. The S. enterica serovars are clinically important due to their significant effect on human morbidity and mortality worldwide (Crump et al., 2004, Majowicz et al., 2010). S. enterica serovar Typhimurium is associated with self-limiting gastroenteritis in immunocompetent individuals and bacteraemia in the immunocompromised, whereas S. enterica serovar Typhi causes typhoid fever which is an acute illness characterized by high fever and abdominal pain. Both serovars have a considerable worldwide impact (Crump et al., 2004, Majowicz et al., 2010, Havelaar et al., 2015, Kirk et al., 2015). Approximately 21.7 million cases of typhoid fever are diagnosed annually, leading to ~433,000 deaths per year and non-typhoid Salmonella causes approximately 93.8 million cases of gastroenteritis annually, leading to ~155,000 deaths per year (Crump et al., 2004, Mogasale et al., 2014, Majowicz et al., 2010). In Canada, non-typhoid Salmonella is the third highest cause of foodborne illness and death (behind Norovirus and Listeria monocytogenes) and second highest contributor to foodborne hospitalizations (Thomas et al., 2015).

In addition to its significant clinical impact, *S*. Typhimurium is widely considered an important model organism for the study of intracellular pathogens. There are a broad range of molecular and cell biology-based tools developed to study its pathogenesis. *S*. Typhimurium is also a natural pathogen of mice, which allows for the elucidation of host-pathogen interactions through infection of inbred and immunodeficient mouse strains. Unfortunately, the study of *S*. Typhi is

largely restricted to cell culture-based experiments due to its limited host range as a human adapted-pathogen. Thus, most studies discussed in this chapter have used *S*. Typhimurium (from here denoted as *Salmonella*) to understand its interactions with host cells.

Natural Salmonella infection occurs through the ingestion of contaminated water or food products. Salmonella uses its Salmonella pathogenicity island-1 (SPI-1) type three secretion system (T3SS) to breach the intestinal mucosa, preferentially in the terminal ileum by targeting the microfold (M) cells that overlie Peyer's patches (Clark et al., 1994, Jones et al., 1994). It induces membrane ruffling and direct invasion of M cells and intestinal epithelial cells (IECs) (Keestra-Gounder et al., 2015, LaRock et al., 2015). Macropinocytic uptake of Salmonella occurs rapidly, with the invading bacteria initially residing in a phagosomal compartment derived from the endocytic pathway termed the 'Salmonella-containing vacuole' (SCV) (Steele-Mortimer et al., 1999, Drecktrah et al., 2007). Inside the SCV, Salmonella is exposed to an acidic pH as well as decreased nutrient availability (limiting amounts of inorganic phosphate) which triggers the expression of Salmonella's second T3SS, SPI-2 (Beuzon et al., 1999, Cirillo et al., 1998, Lober et al., 2006). Salmonella survives within the SCV through the injection of numerous SPI-2 translocated effectors that functionally impact a wide variety of host cellular processes such as preventing lysosomal fusion with the SCV, modulation of the host cytoskeleton and SCV membrane integrity, as well as immune signaling interference. Recently it has been recognized that a subpopulation of *Salmonella* escapes the SCV and replicates within the host cell cytosol (Malik-Kale et al., 2012, Knodler et al., 2010). However, the permissiveness of the cytosol to Salmonella growth depends on the cell type infected. In cultured IECs, Salmonella can escape the nascent SCV and hyper-replicate to reach levels of >100 bacteria per

a cell (Birmingham *et al.*, 2006, Cemma *et al.*, 2011, Knodler *et al.*, 2010, Knodler *et al.*, 2014). Similar levels of cytosolic *Salmonella* were also observed in embryonic fibroblasts by 4 hours post invasion (Birmingham *et al.*, 2006). However, considerably fewer cytosolic *Salmonella* were observed in bone-marrow derived macrophages (BMDMs), likely due to a more hostile (antimicrobial) environment within these cells (Meunier *et al.*, 2014). This particular intracellular lifestyle exposes *Salmonella* to detection by inflammasomes which have been identified as playing a key role in the early host response to *Salmonella* by both professional immune cells and IECs.

1.2 Inflammasomes

Inflammasomes are macromolecular cytoplasmic complexes that function as platforms for the activation of the inflammatory caspases. These complexes promote the processing of the proinflammatory cytokines interleukin-1 β (IL-1 β) and IL-18, as well as the induction of a specialized form of inflammatory cell death termed pyroptosis (Man and Kanneganti, 2015, Storek and Monack, 2015, Stowe *et al.*, 2015, Bergsbaken *et al.*, 2009, Cookson and Brennan, 2001). Since the initial characterization of inflammasomes in 2002, major strides have been made in this field including the discovery of multiple inflammatory caspases; caspase-1, -4, -5, - 11, and -12 (Martinon *et al.*, 2002, Man and Kanneganti, 2015). Caspase-1, -4, -5 and 12 are expressed by primates, while caspase-1 and -11 are expressed in mice (caspase-11 is an ortholog of caspase-4 and -5) (Lamkanfi *et al.*, 2002, Stowe *et al.*, 2015). Researchers have also identified several pattern recognition receptors (PRRs) that upon recognizing their respective cytosolic pathogen-associated molecular patterns (PAMPs) or specific cellular stress or danger molecules (DAMPs), trigger the activation of these caspases. The components that comprise the inflammasome are located intracellularly and in the case of bacterial infections, only activated when a bacterial pathogen has compromised the sterility of the host's cytoplasm, introducing bacterial products or damaging cellular homeostasis. Modulation of the inflammasome is critical because once it is activated, a chain of events is triggered that primes the cell and surrounding tissues to produce a pro-inflammatory response that can result in the rapid self-destruction of the compromised cell (Fink and Cookson, 2006).

Inflammasome activation is classified into two pathways; the canonical inflammasome and the non-canonical inflammasome. The canonical inflammasome consists of three components: a nucleotide-binding oligomerization domain-like (NOD) receptor (NLR) PRR, an adaptor protein bridge and caspase-1 (Storek and Monack, 2015). Upon ligand recognition, the PRR sensor associates with the adaptor protein via their shared domains. This prompts the recruitment of caspase-1 to the adaptor protein-PRR sensor complex through activation of the 'caspase activation and recruitment domain' (CARD) present in both the adaptor protein and caspase-1, which results in the formation of the inflammasome. Caspase-1 exists as a full-length zymogen under homeostatic conditions. The assembly of the inflammasome is presumed to autocatalytically activate caspase-1 through the proteolytic processing of its pro- domain, which in turn cascades to the processing of pro-IL-1 β , pro-IL-18 and gasdermin D (GsdmD) into their active forms, and the induction of pyroptosis (Stowe *et al.*, 2015, Man and Kanneganti, 2015, Storek and Monack, 2015, Bergsbaken *et al.*, 2009).

The non-canonical inflammasome utilizes caspase-4/5 or -11, and while it has been shown to promote pyroptosis and the processing/secretion of IL-1 β and IL-18, its exact functions as well

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as the interplay between itself and caspase-1 remains controversial (Stowe *et al.*, 2015). The unifying feature of the inflammatory caspases is that each contains a CARD at their N-termini that mediates their protein-protein interactions and subsequent activation (Storek and Monack, 2015). Surprisingly, a recent report suggested that caspase-11, as well as caspase-4 and 5, do not associate with a lipopolysaccharide (LPS)-sensing PRR, but instead directly bind LPS via their CARDs, effectively making caspase-4/5/11 their own upstream sensors (Shi *et al.*, 2014). This novel finding requires further examination as it pivots away from the typical PRR-centric model, where specialized sensors are required to activate downstream enzymes via a complex signaling pathway, to an atypical response where the sensors themselves detect and respond to pathogenic stimuli.

Inflammasomes are vital in the restriction of a *Salmonella* infection. Raupach and colleagues (2006) observed significantly higher *Salmonella* burdens in the Peyer's Patches, mesenteric lymph nodes, and spleens of both *Casp1/11^{-/-}* and *Il1\beta/Il18^{-/-}* mice as compared to C57BL/6 mice, five days after oral infection (Raupach *et al.*, 2006). It is important to note that expression of inflammasomes is conserved among different cell types and they are utilized by a variety of cells for innate defense. Until recently, the focus of the inflammasome field has largely been on monocytes and macrophages as the primary effectors of inflammasome-mediated restriction of *Salmonella*. However, recent evidence has revealed many additional cell types utilize inflammasomes to combat intracellular *Salmonella*, and although identical signaling pathways are utilized, the inflammasome-induced phenotypes appears specific to each cell type.

1.2.1 Salmonella and the immune cell inflammasome

1.2.1.1 Macrophages

Classical immunology divides macrophages into two broad cellular subsets: classically activated (M1) or alternatively activated (M2) (Martinez and Gordon, 2014). These designations were assigned to mimic Th cell nomenclature where Th1-derived IFNγ signaling promoted cellular defense against intracellular infections while Th2-driven IL-4 mediated protection against extracellular parasitic infection. Exposure to *Salmonella* PAMPs shifts macrophages typically into the M1 activation status.

After breaching the epithelial barrier, *Salmonella* is engulfed by nearby macrophages or it can induce its own uptake via its SPI-1 T3SS. *Salmonella* ultimately activates the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) signaling pathway in these cells through its detection by Toll-like receptors (TLRs), and/or through SPI-1 translocated effectors activating the mitogen-activated protein kinase (MAPK) pathway (Gurung *et al.*, 2012, Yang *et al.*, 2014, Tam *et al.*, 2008). This triggers secretion of IL-6 and tumor necrosis factor alpha (TNF- α) as well as the expression of pro-IL-1 β and pro-IL-18 (LaRock *et al.*, 2015, Man *et al.*, 2013). Once inside the cytoplasm, *Salmonella* dampens the host pro-inflammatory response by residing in the SCV, shielded from inflammasome detection and shifts its expression from SPI-1 to SPI-2 T3SS effectors. However, the SCV is under constant assault by host molecular pathways attempting to destabilize it as well as induce its fusion with lysosomes (LaRock *et al.*, 2015, Keestra-Gounder *et al.*, 2015). If the SCV is breached and *Salmonella*-inflammasome activating PAMPs become cytoplasmic, this triggers inflammasome formation. The inflammasome can also assemble in response to cellular stress or danger molecules induced by *Salmonella*'s intracellular existence. Once an inflammatory caspase is activated, this triggers processing of the pro-forms of IL-1β and IL-18 as well as rapid cell lysis. As a result, intracellular *Salmonella* are released into the extracellular space, which can benefit both host and pathogen. For the host's benefit, extracellular *Salmonella* are especially vulnerable to responding neutrophils, which can rapidly clear the infection through the generation of reactive oxygen species (ROS) (Keestra-Gounder *et al.*, 2015). However, extracellular release can also provide *Salmonella* with basolateral entry to the intestinal epithelium or access to liver and spleen tissues if the infection become systemic (Mastroeni *et al.*, 2009). Therefore, inflammatory caspase activation must be tightly regulated and inflammasome formation is likely kept as a measure of last resort, since activation ultimately ends with the cell's death.

Salmonella-induced inflammasome formation and subsequent caspase-1 activation in macrophages is mainly orchestrated by the PRRs; Naip and Nlrp3 (Figure 1.1). In mice, Naip has multiple homologues that are able to detect either flagellin (Naip5 and Naip6) or T3SS components PrgJ and PrgI (Naip2, Naip1), while humans express only a single Naip, hNAIP, which responds to PrgI (Storek and Monack, 2015, Man and Kanneganti, 2015). Once stimulated, Naip associates with the adapter protein Nlrc4 which triggers caspase-1 activation. Intracellular *Salmonella* are also detected by the PRR Nlrp3 which identifies molecules associated with cellular stress or danger such as host cell potassium efflux, calcium influx, extracellular ATP, mitochondrial reactive oxygen species, mitochondrial DNA and the translocation of cardiolipin from the inner mitochondrial membrane to the outer membrane (Storek and Monack, 2015, Man and Kanneganti, 2015). Both Naip and Nlrp3 contain different protein sensing, recruitment and activation domains which impact how they interact with caspase-1. Naip associates with the adaptor Nlrc4 via leucine rich repeat domains, while Nlrp3 binds Asc via their shared pyrin domains. Both Nlrc4 and Asc associate with caspase-1 via their individual CARD domains, separately forming the functional inflammasome and activating caspase-1.



Figure 1.1 PAMP and DAMP activation of canonical and non-canonical inflammasomes.

A. Proteins that comprise the inflammasome as illustrated by their respective protein domains. B. Activation of inflammasome signaling pathways in response to *Salmonella* infection. Abbreviations: NLRP3 - NOD-like receptor family, pyrin domain containing 3; NLRP1b - NOD-like receptor family, pyrin domain containing 1; NAIP1-6 - neutronal apoptosis inhibitor protein 1-6; NLRC4 - NOD-like receptor family, caspase-activating and recruitment domain containing protein 4; AIM2 - absent in melanoma 2; ASC - apoptosis-associated speck-like protein; PYD – pyrin; NACHT - NAIP, C2TA (MHC class 2 transcription activator), HET-E (incompatibility locus protein from Podospora anserina) and TP1 (telomerase-associated protein); LRR - leucine rich repeat; FIIND – function to find domain; CARD - caspase activation and recruitment domain; BIR - Baculovirus Inhibitor of apoptosis protein repeat; HIN200 - hemopoietic expression, interferon-inducibility, nuclear localization; mtDNA - mitochondrial DNA; ROS - reactive oxygen species; T3SS - type three secretion system; Casp - caspase; IL - interleukin; K+ - potassium; Ca2+ - calcium; LPS – lipopolysaccharide.
1.2.1.1.1 Naip1-6, hNAIP and Nlrc4

Salmonella is a motile pathogen and produces six to eight peritrichous flagella in order to move through its environment and reach the intestinal mucosal surface where it invades host cells (McCarter, 2006, Fabrega and Vila, 2013). The flagellum consists of a basal body, an anchoring hook component, and a long flagellin filament made up of repeating FliC and FljB structural subunits. In a murine *Salmonella* BMDM infection model, IL-1 β secretion by infected cells is dramatically reduced when *Salmonella* lacks FliC or when their FliC C-terminus amino acid composition is altered (Franchi *et al.*, 2006, Miao *et al.*, 2006). This sensing takes place via the direct intracellular detection of FliC by the PRRs Naip5 and Naip6 in mice (Lightfield *et al.*, 2008, Zhao *et al.*, 2011, Kofoed and Vance, 2011). FljB also induced inflammasome-mediated cytotoxicity at similar levels to a FliC-driven response (Miao *et al.*, 2006). However, there was no significant difference between secreted IL-1 β levels comparing an *S*. Typhi *fliC* mutant to a double *S*. Typhimurium *fljBfliC* mutant, indicating FliC alone is sufficient for inflammasome formation (Winter *et al.*, 2015).

Out of the murine Naips, Naip5 and Naip6 have the highest amino acid sequence identity (94.7%). Naip5 expression in primary macrophages far surpasses that of Naip6, suggesting these receptors may not play equal roles in inflammasome signaling (Wright *et al.*, 2003). Both Naip5 and Naip6 act as co-receptors for Nlrc4 binding, inducing the activation of caspase-1 in BMDMs. Interestingly, there appears to be a functional link between Nlrc4 signaling and actin turnover in macrophages. Actin polymerization is required for Nlrc4-dependent intracellular bacterial burdens, inflammasome assembly, pyroptosis and IL-1β production (Man *et al.*, 2014a). Man and colleagues report this Nlrc4-induced cellular stiffness restricts intracellular replication

and *Salmonella* dissemination throughout tissues. Murine Nlrc4 is also an interesting adaptor because it links caspase-1 activation with the detection of multiple structurally different PAMPs. In addition to associating with the flagellar-detecting Naip5 and Naip6, Nlrc4 also binds to the sensors Naip2 and Naip1 which recognize the T3SS rod and needle apparatus respectively.

Inflammasomes can also detect Salmonella via the recognition of its SPI-1 T3SS. The T3SS is a molecular syringe-like apparatus that punctures the host cell membrane, forming a continuous channel between the bacterium and host, allowing bacterial effectors to be pumped directly into the host cell cytoplasm. Salmonella contains two T3SS and their expression is tightly controlled based on the stage of Salmonella's pathogenesis as well as the detection of specific environmental cues from the gut and within host cells. The SPI-1 T3SS is expressed during the initial invasion of host cells, inducing bacterial-mediated endocytosis. Its various translocated effectors such as SopB, SopE and SopE2, activate the small GTPases; Cdc42 and Rac-1, through their guanine nucleotide exchange factor activity (LaRock et al., 2015). This causes alterations in the actin cytoskeleton and disrupts tight junctions between IECs, producing conditions that facilitate Salmonella's invasion of IECs as well as a generalized weakening of the overall epithelial barrier. Once Salmonella has undergone endocytosis, it alters its vacuole maturation through its SPI-2 T3SS. While not as abundantly expressed as the SPI-1 T3SS, the SPI-2 T3SS injects effectors across the SCV membrane, yielding a relatively safe replication niche for Salmonella by altering vacuolar membrane and trafficking to limit lysosomal fusion as well as rerouting vital metabolites to be imported into the SCV (Figueira and Holden, 2012).

Initially the discovery that the Salmonella SPI-1 T3SS inner rod protein (PrgJ) also triggered a Nlrc4 inflammasome-dependent response was confusing because of the protein structural differences between PrgJ and FliC. Zhao and colleagues discovered flagellin and Naip5 coimmunoprecipitated with Nlrc4, but neither Naip1 nor Naip2 coimmunoprecipitated in the presence of flagellin (Zhao et al., 2011). In a yeast two hybrid assay probing for associations with the Burkholderia thailandensis T3SS rod protein BsaK, the authors uncovered an interaction between BsaK and Naip2. They confirmed Naip2 detected the T3SS rod protein, through infection of BMDMs with either Salmonella $\Delta fliC/fljB$ or $\Delta fliC/fljB/prgJ$ mutants, to remove the confounding caspase-1 activation from flagellin detection by Naip5/6. The presence of PrgJ produced cleaved, activated caspase-1 after 30 minutes of infection that was eliminated following a siRNA knockdown of Naip2. Also, no cleaved caspase-1 was detected from the cells infected with the $\Delta fliC/fljB/prgJ$ strain. Further studies revealed that the flagellin and T3SS rod protein's ability to stimulate Naip activity was localized to their C-terminal leucine-rich helical hairpin regions (Lightfield et al., 2008, Poyraz et al., 2010, Miao et al., 2010). This is of special interest to studies of Salmonella because the amino acid composition of its SPI-1 (PrgJ) and SPI-2 (SsaI) T3SS inner rod proteins differ at these residues. SsaI encodes several amino acid substitutions compared to PrgJ, but most notably it displays a switch from valine to leucine at its C-terminus (Miao et al., 2010). When Salmonella grown under SPI-2 inducing conditions were used to infect BMDMs, the infected cells did not secrete IL-1 β after 8 hours of infection, indicating an absence of inflammasome activation. Interestingly, swapping the eight amino acids at the C-terminus of SsaI with those from PrgJ restored Nlrc4 binding and IL-1 β secretion, uncovering a potential strategy utilized by intracellular Salmonella to evade the inflammasome.

In humans, only one Naip orthologue exists (hNAIP) and although it has high sequence identity to murine Naip5 (68% amino acid identity), it does not recognize or respond to flagellin (Zhao et al., 2011, Storek and Monack, 2015). In phorbol myristate acetate-stimulated U937 and THP-1 human macrophages, hNAIP was shown to respond to PrgI (the T3SS needle proteins from SPI-1) but not PrgJ (Yang *et al.*, 2013). This finding was of significant interest because murine Naip activity against the T3SS needle protein had not previously been detected. Yang and colleagues proposed that murine NIrc4 was indeed capable of responding to intracellular PrgI but the activity(s) of Naip2 and Naip5 shielded its effects (Yang et al., 2013). To this end, they delivered the needle protein directly into the cytoplasm of BMDMs utilizing the LFn-PA delivery system (this consists of a fusion of needle proteins to the N-terminal domain of Bacillus anthracis lethal factor (LFn) which mediates cytosolic delivery through the anthrax protective antigen (PA) channel) (Yang et al., 2013, von Moltke et al., 2012). This triggered robust caspase-1 activation, which was completely abolished in *Nlrc4*-deficient BMDMs (Yang et al., 2013). Furthermore, when HEK293T cells were co-transfected with Nlrc4, Naip1 and PrgI, large oligomeric complexes containing all three components could be resolved by native polyacrylamide gels, confirming Naip1 was an active detector of the T3SS needle protein and that it activated caspase-1 through Nlrc4 binding.

This difference between the number of Naips between human and mice as well as the specificity of detecting a single PAMP by hNAIP could reflect a control mechanism for the intracellular sensing of bacteria. In mice, both Naip2 and Naip5 are highly expressed in systemic organs such as the spleen, whereas Naip1 could not be detected (Yaraghi *et al.*, 1999, Wright *et al.*, 2003). These differences in expression could explain the overall responsiveness of murine macrophage

inflammasomes to endogenous PrgJ and FliC from *Salmonella*, whereas PrgI must be artificially injected into cells to activate caspase-1 (Yang *et al.*, 2013). However, hNAIP, like Naip1, detects PrgI. Compared to flagellin (which is downregulated after *Salmonella*'s internalization) and the PrgJ inner rod protein (which remains sheathed within the T3SS unit), it is more likely that the host cell cytosol could be exposed to PrgI during an active *Salmonella* infection. Limiting the number of PAMPs that can activate the inflammasome and restricting activation to PAMPs that are only detected once the *Salmonella* has successfully infected and replicated within the host cells may represent a novel strategy by *Salmonella* to limit inflammasome activation until it is too late to disrupt infection. Little information is currently available regarding how inflammasome responses are terminated or if they can be terminated at all.

1.2.1.1.2 Nlrp3 and Asc

Inflammasome stimulatory PAMPs such as flagellin and T3SS structural proteins are essential bacterial components for the pathogenesis of *Salmonella* and are readily detected by the Naip-Nlrc4 inflammasome. To shield itself from constant assault by host cell molecular processes and continue its intracellular life cycle, *Salmonella* can alter its PAMPs by decreasing expression of flagellin and switching from its SPI-1 to its SPI-2 T3SS. To combat this evasion strategy, the host cell utilizes the Nlrp3 inflammasome, which instead of detecting PAMPs, responds to increased levels of host cell-derived stress and danger molecules.

Studies of the Nlrp3 inflammasome across a wide range of bacterial infections has uncovered its responsiveness to host cellular stress molecules such as excessive potassium efflux, calcium influx, extracellular ATP, mitochondrial reactive oxygen species and DNA and the translocation

of cardiolipin from the inner mitochondrial membrane to the outer membrane. The mechanism by which Nlrp3 can detect such a wide range of structurally and chemically unrelated stimuli is still under investigation. In the case of Salmonella interactions with macrophages, Nlrp3 is responsible for activation of the inflammasome independently of SPI-1 and SPI-2 T3SS and is tied to alterations in the host cell most likely induced due to intracellular Salmonella metabolism (Broz et al., 2012, Gurung et al., 2012, Rathinam et al., 2012, Sanman et al., 2016). During a screen of Salmonella genes that modulate Nlrp3 activation, Wynosky-Dolfi and colleagues identified four genes; acnB – aconitase, which mediates conversion of citrate to isocitrate in the tricarboxylic acid cycle (TCA); bcfB – fimbrial chaperone; rcsD – a two component system which controls capsule and flagellar synthesis; and melB - a symporter of melibiose and monovalent cations (Wynosky-Dolfi et al., 2014). The authors focused on the link between TCA cycle disruption and Nlrp3 activation by constructing mutants deficient in aconitase or isocitrate dehydrogenase. Mice infected with *acnB* mutants displayed significantly higher serum levels of IL-18 as compared to mice infected with wildtype *Salmonella* and these levels were reduced in $Casp 1/11^{-/-}$ and $Nlrp 3^{-/-}$ mice. Mutation of these bacterial TCA cycle enzymes in conjunction with flagellar proteins, led to high intracellular citrate levels and elevated levels of mitochondrial ROS, which resulted in rapid Nlrp3-dependent, Nlrc4-independent inflammasome activation. Limiting the resulting mitochondrial ROS (mROS) levels in murine BMDMs through pharmacological inhibitors or through the use of mitochondrial catalase transgenic mice eliminated Nlrp3 inflammasome activation by the Salmonella TCA cycle mutants, indicating that Salmonella specifically activated the Nlrp3 inflammasome through mROS production.

Nlrp3 based caspase-1 activation occurs through the binding of these two molecules to the adaptor protein Asc. Moreover, Asc expression is crucial for Salmonella-induced IL-1ß and IL-18 secretion by murine macrophages (Broz et al., 2010, De Jong et al., 2014). The physiological importance of Asc-containing foci in Naip-Nlrc4 inflammasome activation is still under investigation. The CARD of Nlrc4 directly interacts with the CARD of caspase-1, therefore T3SS and flagellin-inflammasome signaling should occur independently of Asc foci formation. However, Asc foci were detected in Salmonella infected Nlrp3^{-/-} deficient murine BMDMs and this aggregation was found to be dependent on the presence of flagellin (Broz et al., 2010). Proell and colleagues explored this phenotype through a bimolecular fluorescent complementation system, which allowed them to visualize Asc foci within a living cell (Proell et al., 2013). The CARDs of NIrc4 and caspase-1 were fused with two complementary fragments of the fluorescent protein Venus. These fragments do not reconstitute spontaneously, but a functional fluorescent signal is produced when Nlrc4 and caspase-1 associate. When the CARDs of NIrc4 and caspase-1 were co-expressed in HEK293T cells, this produced a diffuse signal throughout the cell. However, when full-length Asc was introduced, tight punctuate foci were formed, suggesting that Naip-Nlrc4-caspase-1 inflammasomes could utilize Asc as a platform for formation. Further support for this model is given through co-localization experiments performed by Man and colleagues (Man *et al.*, 2014b). The authors observed multiple NLRs, such as Nlrp3 and Nlrc4, co-localizing with Asc specks. They propose that Salmonella PAMPs activate NLRs which associate, thereby forming a nucleation point for the assembly of an Asc foci. This in turn leads to the recruitment of caspase-1, producing a functional inflammasome. There is also the potential that Asc might play a role in inflammatory signal amplification. Once infected macrophages undergo pyroptosis and lyse, researchers found that Asc aggregates appear to

remain active in the extracellular space (Storek and Monack, 2015). These aggregates can be phagocytosed by nearby cells, leading to the activation of their own inflammasomes and the subsequent cleavage and secretion of IL-1 β and IL-18. As a result, Asc foci released by pyroptotic cells may play a key role in amplifying inflammatory signals by providing a platform for inflammasome formation.

1.2.1.1.3 Caspase-11

Detection of cytosolic *Salmonella* LPS can also induce inflammasome-mediated cell death through the actions of caspase-11. LPS must enter the cytosol before it can activate the caspase-11 inflammasome, in the case of *Salmonella*, this means the bacterium must be directly exposed to the cytoplasm, not contained inside the SCV. Experimentally this can be induced through direct microinjection or transfection of purified LPS into the cytoplasm, or the use of a $\Delta fljAB\Delta fliC\Delta sifA$ Salmonella mutant, which does not express flagellin and upon infecting a host cell, readily escapes the SCV. SifA is a SPI-2 translocated effector which is vital for maintaining the stability of the SCV (Beuzon *et al.*, 2000, Cirillo *et al.*, 1998). Utilizing this *Salmonella* mutant, Aachoui and colleagues demonstrated its enhanced clearance by caspase-11 driven pyroptosis was independent of Nlrp3, Nlrc4 and Asc (Aachoui *et al.*, 2013).

Once caspase-11 has detected LPS, this triggers its own proteolytic activation inducing pyroptosis and the cleavage and secretion of IL-1 β and IL-18. A group based out of Genentech reported that caspase-1 is dispensable for caspase-11 mediated pyroptosis, but caspase-11 dependent IL-1 β processing requires Nlrp3, Asc and caspase-1 (Kayagaki *et al.*, 2011, Kayagaki *et al.*, 2013, Baker *et al.*, 2015, Ruhl and Broz, 2015, Schmid-Burgk *et al.*, 2015). The reduced

IL-1 β secretion by *Casp11^{-/-}* cells is not due to lower expression of pro-IL-1 β as both wildtype and *Casp11^{-/-}* deficient BMDMs expressed similar pro-IL-1 β levels when infected with *Salmonella* (Broz *et al.*, 2012). Recently Casson and colleagues observed that the human orthologue of caspase-11, termed caspase-4, mediated IL-1 α secretion and cell death in response to *Salmonella* infection of primary human macrophages (Casson *et al.*, 2015). They also noted that caspase-5 was not processed during the infection and did not appear to play a role in host restriction of intracellular *Salmonella*.

The interplay between canonical and non-canonical inflammasomes remains poorly understood. Caspase-1 and caspase-11 can interact but it is unclear whether the two caspases form a catalytically active heterocomplex or if proximity of the two caspases within a cell is sufficient for caspase-1 and -11 co-activation. Caspase-11's major role appears to be in the initiation of pyroptosis. Pyroptosis is responsible for the clearance of intracellular bacteria through the self-directed destruction of the infected cell. This destroys the protected niche established by the pathogen within the host cytosol, exposing the previously intracellular *Salmonella* to various extracellular defense mechanisms, including neutrophils. Pyroptosis, rather than the release of IL-1 β and IL-18, has been proposed to be the dominant process underlying caspase-11's key role in the LPS-induced lethal sepsis model (Kayagaki *et al.*, 2011). Mice deficient in *Casp1/11^{-/-}*, or both *IL-1\beta/IL-18^{-/-}* were shown to rapidly succumb to high dose injections of LPS, whereas *Casp11^{-/-}* mice remained resistant. However, the mechanism of pyroptosis and the cell types responsible for *in vivo* lethal sepsis are still under investigation.

Caspase-11 readily detects cytosolic Salmonella but it can also detect SCV-protected Salmonella once the vacuole is compromised (Broz et al., 2012, Rathinam et al., 2012). Meunier and colleagues explored the requirement for Trif-dependent production of type-I interferons and caspase-11 driven cell death by Gram-negative bacteria (Meunier et al., 2014). Through a proteomics-based expression analysis for proteins highly induced upon Salmonella infection, they identified the interferon-induced GTPases as the most strongly upregulated proteins. In particular, they observed the large 65-67 kDa guanylate-binding proteins (GBPs) as being highly expressed. They then moved to examine the interaction of these GBPs during a Salmonella infection and detected guanylate-binding protein 2 (GBP2) co-localizing with the SCV in murine BMDMs and its expression led to higher cytosolic populations of Salmonella (Meunier et al., 2014). The expression of GBP2 was also linked to caspase-11 based cytotoxicity in response to Salmonella infection, while overall depletion of the GBPs led to a significant decrease in IL-1 β secretion. This suggests the GBPs destabilize the SCV, by an as yet unknown mechanism, leading to the release of *Salmonella* into the cytosol, exposing them for detection by both the canonical and non-canonical inflammasomes. The authors also observed that if cytosolic Salmonella were targeted by autophagy, this significantly reduced caspase-11 activation (Meunier et al., 2014).

1.2.1.1.4 Other inflammasomes involved in *Salmonella* detection

The NLRP6 and NLRP12 inflammasomes have been linked to the maintenance of intestinal homeostasis, primarily in the context of chemical-induced colitis (Zaki *et al.*, 2010, Elinav *et al.*, 2011). Studies have observed that NLRP6 and NLRP12 suppress colon inflammation through the inhibition of NF-κB signaling (Zaki *et al.*, 2011, Allen *et al.*, 2012). In terms of *Salmonella* i.p.

infection, both *Nlrp6^{-/-}* and *Nlrp12^{-/-}* mice were shown to carry significant lower bacterial burdens in their spleens and livers (Zaki *et al.*, 2014, Anand *et al.*, 2012). Moreover, both *Nlrp6^{-/-}* and *Nlrp12^{-/-}* BMDMs were found to produce elevated levels of TNF- α , IL-6 and KC in response to bacterial infection but showed no difference when compared to wildtype cells in terms of IL-1 β and IL-18 expression. The authors also observed higher numbers of circulating monocytes and neutrophils in this study (Anand *et al.*, 2012). While the ligand that the NLRP6 and NLRP12 inflammasomes detect has not yet been identified, over-expression studies suggest NLRP6 association with Asc and caspase-1 results in IL-1 β secretion, and *Salmonella* LPS alone was able to induce NLRP12-mediated inhibition of NF- κ B (Grenier *et al.*, 2002, Zaki *et al.*, 2014). Therefore, the NLRP6 and NLRP12 inflammasomes may play immune dampening roles in response to specific stimuli, and this suppression might be exploited by *Salmonella* to modulate host antimicrobial responses as a means to promote their prolonged persistence and survival within infected hosts.

One of the strongest *Salmonella* inflammasome stimulants is the SPI-1 translocon protein SipB, which activates caspase-1 (Hersh *et al.*, 1999). Deletion of *sipB* causes a severe deficiency in inflammasome activation within murine BMDMs which is independent of the number of intracellular bacteria with these cells (Storek and Monack, 2015, Wynosky-Dolfi *et al.*, 2014). The initial characterization of SipB via affinity purification uncovered its interaction with caspase-1, but it is unclear if this occurs through direct binding, or alternatively, via an interaction with a PAMP or DAMP sensor. During the initial stages of *Salmonella* invasion, SipB associates with the *Salmonella* proteins SipC and SipD to assemble a channel on the distal tip of the needle complex, that spans the host cell plasma membrane and facilitates passage of

T3SS effectors (Galan, 1998). It is also possible that SipB signals through the Nlrp3 inflammasome through cellular stress molecules induced upon host membrane damage. However, this does not explain the direct binding of SipB to caspase-1 and indicates further study is required to uncover the underlying mechanism.

1.2.1.2 Dendritic cells (DCs)

The majority of inflammasome research has centered on murine macrophages however recent studies suggest inflammasomes are active in multiple cell types and each cell type may be unique in its cellular response to *Salmonella* inflammasome activation (Figure 2).





DCs have increased *Naip1* transcript levels and their activation prompts the secretion of IL-18, which directly signals memory CD8+ T cells to secrete IFN- γ . *Salmonella* triggers both Naip and Nlrp3 inflammasome activation in neutrophils resulting in IL-1 β and IL-18 secretion. However, neutrophil inflammasome activation does not elicit pyroptosis producing a cellular source of sustained IL-1 β . *Salmonella* also activates an epithelial cell-intrinsic inflammasome through the Naip-Nlrc4 axis as well as the non-canonical inflammasome, caspase-11. Activation produces IL-18 secretion and IEC extrusion from the epithelium, however no IL-1 β is produced by IECs. Abbreviations: *Naip* neuronal apoptosis inhibitor protein; *Nlrc4* NOD-like receptor family, caspase-activating and recruitment domain containing protein 4; *Nlrp3* NOD-like receptor family, pyrin domain containing 3; *Asc* apoptosis-associated speck-like protein; *IL-1\beta* interleukin 1 beta; *IL-18* interleukin 18; *IFN-* γ interferon-gamma; *IEC* intestinal epithelial cell.

Both macrophages and dendritic cells are phagocytic cells critical in the immune response to Salmonella infection. The primary function of DCs is to process antigens and present them to naïve T-lymphocytes. Early work by Fink and colleagues established that Salmonella infection of murine BMDCs triggers a caspase-1 dependent cell death (Fink et al., 2008). In an intravenous mouse model of Salmonella infection, the authors observed activation of the Naip-Nlrc4-caspase-1 inflammasome (Kupz et al., 2012). Activation prompted the secretion of IL-18, which directly signaled memory $CD8^+$ T cells to secrete IFN- γ . This interaction is important in highlighting that DC-T cell interactions are not limited to simple antigen-specific responses. DCs can also orchestrate innate immune responses through inflammasome-directed cytokine secretion. Interestingly, the majority of murine DC inflammasome signaling appears to utilize Naip5 and Naip1. DC based Naip2 sensing of PrgJ was not required for the early regulation of innate IFN- γ secretion by memory CD8⁺ T cells. Yang and colleagues observed that primary murine BMDCs and immortalized DC2.4 cells produce higher levels of Naip1 transcripts compared to BMDMs (Yang et al., 2013). When DC2.4 cells were stimulated via transfection with plasmids encoding T3SS needle transcripts, this triggered a more robust caspase-1 activation than in BMDM cells and this activation was silenced following siRNA knockdown of *Nlrc4* or *Naip1*.

There has also been a preliminary study of *Salmonella* infection of human monocyte-derived DCs (Dreher *et al.*, 2002). The resulting inflammatory response was dependent on SipB expression and resulted in caspase-1 activation and the release of IL-18.

1.2.1.3 Neutrophils

Neutrophils are recruited in large numbers to the site of *Salmonella* infection where they play a key role in clearing the infection. While neutrophils are commonly viewed as cellular targets of IL-1 β , caspase-1 expression by infected hosts is associated with efficient neutrophil mediated clearance of *Salmonella*. Interestingly, recent evidence has established that neutrophils also express inflammasomes, and their activation plays a vital role in the restriction of Salmonella infection (Broz et al., 2012, Chen et al., 2014). Chen and colleagues observed that both human and mouse neutrophils contained transcripts for *Nlrp3* and *Nlrc4*, and in human neutrophils, these mRNA levels were significantly greater than those expressed by BMDMs or BMDCs (Chen et al., 2014). When murine neutrophils and BMDMs were infected with Salmonella, both cell types secreted IL-1 β , albeit 5-fold less for neutrophils. The secretion of IL-1 β was lacking in cells purified from *caspase-1/11^{-/-}* mice and reduced in $Nlrc4^{-/-}$ and $Asc^{-/-}$ mice. Interestingly, neutrophils secreted negligible amounts of IL-18. The authors then moved to in vivo experiments where they depleted neutrophils from mice utilizing α -Ly6G antibodies, which did not alter the abundance of other myeloid cells. The authors then challenged isotype control (mock treated) and neutrophil-depleted mice with *Salmonella* and observed that IL-1 β levels remained at baseline levels in the neutrophil-depleted mice while control mice displayed 3-fold higher IL-1 β levels after 6 hours of infection. Neutrophil depletion did not significantly affect IL-18 levels even after 12 hours of Salmonella infection. Interestingly, although neutrophils display a potent inflammasome response, they were highly resistant to pyroptosis. When the authors quantified intracellular lactate dehydrogenase released into the supernatant as a measure of *in vitro* and *ex* vivo pyroptosis, they reported significantly decreased neutrophil cytotoxicity compared to BMDMs when the cells were infected by *Salmonella*. It appears that neutrophils express a

specialized inflammasome, decoupling pyroptosis from NIrp3 and NIrc4 signaling. The molecular mechanisms underlying pyroptosis remain elusive and the proteins and proteolytic substrates involved are still under intense investigation. Only HMGB1 and GsdmD have been implicated as downstream products from macrophage caspase activation (Kayagaki *et al.*, 2015, Shi *et al.*, 2015). This suggests that inflammasome driven pyroptosis and cytokine activation/secretion can be mechanistically separated, likely through the downregulation or proteolytic removal of a vital substrate required for the lytic cell death pathway. Overall, this suggests an innate defense mechanism for the restriction of *Salmonella* infection. Macrophages secrete IL-1 β to recruit neutrophils and then undergo pyroptosis as a means to limit vacuolar replication of *Salmonella*. This releases the bacteria into the extracellular environment where they are more effectively killed by neutrophils through ROS production (Miao *et al.*, 2010).

1.3 Inflammasome evasion by Salmonella

Once *Salmonella* has breached the host cell membrane, it engages in an intracellular battle for survival with host cell molecular defense pathways. To evade detection by inflammasomes, *Salmonella* can modify its detectable PAMPs through downregulation of PAMP expression, chemical modification of PAMPs, or through the targeting of host cell types with limited inflammasome activity.

1.3.1 PhoP-PhoQ

FliC, PrgJ and PrgI are potent activators of the Naip-Nlrc4 inflammasome in murine macrophages. To evade detection, *Salmonella* represses expression of all three proteins during its intracellular lifecycle through its PhoP-PhoQ regulatory system (Miao *et al.*, 2006). The PhoP-

PhoQ system is expressed when *Salmonella* is intracellular and has proven paramount for the survival of Salmonella within macrophages (Valdivia and Falkow, 1997, Miller et al., 1989, Heithoff et al., 1999, Mouslim and Groisman, 2003). It is also involved in the regulation of SPI-2 expression, alongside other regulatory sensors (Groisman, 2001, Lee et al., 2000, Bijlsma and Groisman, 2005). PhoP-PhoQ strongly represses *fliC* and SPI-1 expression, decreasing the presence of these inflammasome formation-inducing PAMPs (Miao et al., 2006). When Salmonella is engineered to constitutively express flagellin through a plasmid-based expression system where *fliC* expression is driven by *sseJ* promoter (encoded in SPI-2), this significantly increased IL-1 β secretion and caspase-1 cleavage in BMDMs compared to wildtype Salmonella (Miao et al., 2010). Constitutive FliC expression also led to the attenuation of systemic Salmonella infection in mice. Following challenge with wildtype Salmonella, mice succumbed to infection within 6-8 days whereas the P_{SPI2}-*fliC* SPI-2 expressing strain was unable to kill C57BL/6 mice. The hyper-activation of the Naip5/6-Nlrc4 inflammasome is likely responsible for this decrease in pathogenesis and demonstrates that the ability of *Salmonella* to actively regulate PAMP expression plays a key role in its ability to survive in the host intracellular environment.

1.3.2 LPS

Another target of the *Salmonella*-induced inflammasome is LPS. Intracellular LPS stimulates non-canonical inflammasome activity via caspase-11. Recent evidence has suggested the lipid A of LPS binds caspase-11 via its CARD (Shi *et al.*, 2015). LPS binding was shown to induce caspase-11 oligomerization, activation and subsequent pyroptosis by the host cell (Kayagaki *et al.*, 2013). Interestingly, caspase-11 demonstrates decreased activity in response to tetra-acylated

lipid IVa and penta-acylated LPS (Shi et al., 2015, Hagar et al., 2013, Kayagaki et al., 2013). This is independent of the capacity of caspase-11 to bind to these moieties and instead was linked to an inability of caspase-11 to undergo oligomerization once bound to these forms of LPS (Shi et al., 2015). Salmonella can chemically modify its LPS through the PhoP-PhoQ and PmrA-PmrB regulatory systems (Ernst et al., 2001). Interestingly, LPS from Salmonella phoP mutants induces higher TNF-α expression from BMDMs compared to wildtype LPS (Guo et al., 1997). Potential modifications of LPS include reduction in O-antigen length, addition of palmitate to lipid A, addition of phosphate, phosphoethanolamine and/or aminoarabinose to lipid A, or the incorporation or removal of myristate from lipid A (Ernst et al., 2001, Raetz et al., 2007). The lipid A from intracellular wildtype *Salmonella* growing inside the murine macrophage cell line RAW264.7 cells has been shown to be hexa-acylated, and heavily modified with 4-amino-4-deoxy-1-arabinose, phosphoethanolamine, 2-hydroxymyristate, and palmitate (Gibbons et al., 2005). Manipulation of the LPS modification protein machinery alters the immunogenicity of Salmonella LPS. When Salmonella's lipid A is changed into a penta-acylated form through mutation to both *msbB* and *pagP*, this significantly reduced its ability to stimulate the human monocyte cell line MM-6 to secrete IL-1 β and TNF- α compared to both wildtype LPS and single *msbB* mutants, which expressed a mix of both penta- and hexa-acylated LPS forms (Kong et al., 2011). During an oral Salmonella infection of BALB/c mice, pooled serum IL-1a levels were also decreased in response to infection by the double *msbB* and *pagP* mutants as compared to wildtype Salmonella, whereas IFN- γ levels were significantly increased (Kong et al., 2011). This suggests that Salmonella can dampen the host inflammatory response by modifying its LPS, which may impact its ability to associate with, and be recognized by caspase-11. The non-canonical inflammasome field is still in its infancy and the ability of caspase-11 to

bind to different modified forms of *Salmonella* LPS has not been explored. The binding experiments reported by Shi and colleagues were conducted with *Salmonella* lysates produced from boiled overnight cultures, and the structure of the *Salmonella* LPS that activated caspase-11 was not determined (Shi *et al.*, 2015). It is likely that different chemical modifications to *Salmonella*'s LPS directly impacts the ability of caspase-11 to undergo oligomerization and represents another strategy by which *Salmonella* modifies its inflammasome-inducing PAMPs as a means to promote its intracellular survival.

1.3.3 Targeted infectivity

In addition to the intrinsic modifications *Salmonella* utilizes to evade the inflammasome, *Salmonella* can also target cellular niches that exhibit reduced inflammasome activity. Recently, *Salmonella* was discovered to preferentially survive in macrophages that display an M2 phenotype (Eisele *et al.*, 2013, McCoy *et al.*, 2012). This was tied to differences in the cellular metabolic pathways expressed between M1 and M2 macrophages. In particular, the upregulation of the eukaryotic transcription factor PPAR δ in M2 cells, which is involved in sustaining fatty acid metabolism and was shown to directly impact glucose availability to intracellular *Salmonella*, resulting in enhanced bacterial replication (Eisele *et al.*, 2013). However, it is also possible that the persistence of *Salmonella* in this cell type could be tied to the comparatively reduced expression of inflammasome components in M2 macrophages (Storek and Monack, 2015). Jourdan and colleagues observed that obese ZDF rats display high mRNA transcripts of *Nlrp3*, *1l1* β and *1l18*, high IL-1 β and IL-18 protein levels and robust caspase-1 activity (Jourdan *et al.*, 2013). When treated with the cannabinoid inverse agonist JD5037, this induced an M1-to-M2 shift and the authors observed a significant decrease in all three markers of inflammasome function. Another study observed an increase in the number of M2 macrophages in *Nlrp3^{-/-}* deficient mice (Vandanmagsar *et al.*, 2011). These studies suggest that the inflammasomes of M2 macrophages are less active and this could be an additional factor that regulates what makes these cells attractive as a cellular niche for *Salmonella* replication and persistence.

1.4 Salmonella and the intestinal epithelial inflammasome

Interestingly, the specific PRR responsible for downstream caspase activation appears to depend on the route of Salmonella infection. In a systemic Salmonella infection model, where Salmonella primarily encounters myeloid cells, Nlrc4 and Nlrp3 play redundant roles in stimulating caspase-1 activation. Only mice deficient in both Nlrc4 and Nlrp3 demonstrate increased susceptibility to Salmonella infection (Broz et al., 2010). However, in a streptomycin (Strep)-pretreatment orogastric mouse model for Salmonella gastroenteritis, where Salmonella must first breach the intestinal epithelium, this redundancy is not apparent in a susceptible infection model wherein Balb/c mice deficient in only Nlrc4 were found to be more vulnerable to Salmonella infection (Franchi et al., 2012). The authors also observed that functional IL-1 signaling increased mouse survival in a Balb/c Salmonella oral infection but was superfluous in an *i.p.* infection (Franchi et al., 2012). Infection of Nlrp3, Nlrc4 or Il1r deficient mice derived onto a C57BL/6 background showed no difference from wildtype in terms of mouse survival regardless of infection route (Franchi et al., 2012). In contrast, Wynosky-Dolfi and colleagues observed route dependent differences in inflammasome signaling in C57BL/6 background mice (Wynosky-Dolfi et al., 2014). Here, a Salmonella triple mutant (acnBfliCfljB which activates Nlrp3 by increasing bacterial-induced mitochondrial ROS production) was significantly attenuated compared to its *fliCfljB* parental strain in wildtype spleen and liver murine

colonization after oral infection. However, similar levels were observed in caspase-1/11 deficient mice, while no significant difference between strains was observed after i.p. injection into either wildtype or caspase deficient mice (Wynosky-Dolfi *et al.*, 2014). These results suggest that *Salmonella* encounters a unique inflammasome pathway at the intestinal mucosal surface.

1.4.1 The intestinal epithelium

The intestinal epithelium is a single layer of cells that differs greatly in architectural structure as well as cellular composition between the small and large bowel. In the small intestine, the epithelium extends over structures that protrude into the lumen, called villi, thereby increasing the mucosal surface area and subsequent nutrient absorption. Villi are absent from the colon, resulting in a relatively flat mucosal surface that limits potential damage inflicted by semi-solid stool transiting through the large bowel. The epithelium itself is not a straight layer of cells, but instead consists of invaginations termed 'crypts of Lieberkühn' (Spence *et al.*, 2011). Intestinal stem cells reside at the base of these crypts and give rise to transition zone, with the mature IEC eventually shedding into the lumen at the apex of crypts (or villi in the small intestine) (Crosnier *et al.*, 2006). Intestinal crypts undergo constant cycles of IEC replenishment and renewal, and under homeostatic conditions, it is estimated that an entire crypt is replaced every 4 to 5 days (van der Flier and Clevers, 2009).

Various differentiated cell types are found within the mammalian gut epithelium and each carries out unique and specialized functions. The distribution of these cell types is also different between the small and large bowel (Figure 1.3). These cell types include: enterocytes, the most prominent cell type of the intestinal epithelium that is responsible for nutrient and water absorption; various secretory cells such as goblet cells that secrete mucins; enteroendocrine cells that secrete hormones; and Paneth cells that release antimicrobial factors to protect nearby stem cells at the base of small intestinal crypts (Crosnier et al., 2006, Gribble and Reimann, 2016, Rodriguez-Colman et al., 2017, Johansson and Hansson, 2016). Finally, there are the chemosensory tuft cells, which play a key role in defense against helminths, and M cells that are integral to the uptake and eventual presentation of luminal antigens to the immune system (Ohno, 2016, von Moltke et al., 2016, Gerbe et al., 2016, Howitt et al., 2016). In general, the majority of cell types located in the colon are also found in the small intestine, these include the enterocytes (also referred to as colonocytes in the colon), enteroendocrine cells, goblet cells as well as tuft cells, but Paneth cells, which intercalate with stem cells at the bottom of crypts, and M cells, which are located overlying Peyer's Patches appear unique to the small bowel. However, recent reports have observed colonic 'M cell-like' lymph nodes and 'Paneth-like' cells. Interestingly, these 'Paneth-like' cells express Paneth cell markers and their prevalence was found to increase at localized sites of inflammation (Rothenberg et al., 2012, Perminow et al., 2010, Sasaki et al., 2016, Parikh et al., 2019).

(A) Small intestine





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A. Representative small intestinal epithelium. Enterocytes comprise the major cells type found in the crypt–villus axis and are capable of secreting antimicrobial peptides (RegIII γ , β -defensins, cathelicidin). Paneth cells located at the base of the crypt produce specific antimicrobial peptides (lysozyme, α -defensins, sPLA2). M cells are localized to the follicle-associated epithelium (FAE) overlying Peyer's patches and directly participate in antigen uptake and passage to underlying immune cells. Goblet cells also facilitate luminal antigen transfer to dendritic cells via goblet cell-associated antigen passages (GAPs). B. Representative colonic epithelium. Colonocytes and goblet cells are the major cell types of the colonic crypt. Goblet cells are responsible for the formation of the outer and inner mucus layer through secretion of Muc2 mucin as well as other proteins (Agr2, ZG16, CCLA1, RELM β) that are associated with this protective barrier. Printed with permission from Trends in Immunology (2018) Sep;39(9):677-696.

1.4.2 Intestinal epithelial intrinsic inflammasome

Understanding the mechanisms by which IECs defend against enteric pathogens is paramount to our ability to prevent and treat food-borne illnesses. Historically, the role played by IECs in innate defense was defined by their location. A single layer of cells that function as a structural barrier, physically sequestering bacteria and other noxious stimuli within the gut lumen, as a means to prevent their interaction with, and overt stimulation of the underlying immune system. However, we and others have hypothesized that the intestinal epithelium is not a passive barrier, but instead actively responds and defends the host when challenged by pathogenic attack. Upon direct invasion of IEC by intracellular pathogens, a more vigorous means of host defense is triggered, by which enterocytes can physically expel themselves from the intestinal epithelial lining while the remaining cells still maintain mucosal barrier function. This process limits the ability of intracellular pathogens to breach the epithelial barrier, and instead they are extruded into the fecal stream, along with the sloughed enterocyte. This expulsion is independent from the homeostatic process of continuous enterocyte renewal, and recent characterization by our laboratory and others have uncovered that this process is regulated through activation of the inflammatory caspases (caspase-1, -4 and -11) as well as caspase-8 (Knodler et al., 2014, Sellin et al., 2014, Rauch et al., 2017).

1.4.3 IEC intrinsic Naip, Nlrc4 and Caspase-1 axis

In studies designed to define the actions of IEC -intrinsic inflammasomes, Sellin and colleagues focused on the initial stages of *Salmonella* infection where they observed that by 6 hours post-infection (p.i.), the cecum contained numerous infected enterocytes that contained up to 20 densely packed bacteria, predominantly occupying the supranuclear region of IECs (Sellin *et al.*,

2014). These populations, which the authors termed 'microcolonies', were still evident until 12 hours p.i., at which time infected IECs began to be extruded from the epithelial layer and released into the lumen. By 36 hours p.i., the hyper-infected IECs were entirely cleared from the epithelium and only small numbers of IECs containing one to two bacteria remained. In contrast, mice deficient in *Naip1-6* or *Nlrc4* displayed much higher densities of *Salmonella* in their intestinal epithelium (~50- to 100- fold higher load), with these loads persisting for up to 36 hours p.i., indicating the mechanism responsible for infected IEC extrusion was tied to caspase-1 activation by Nlrc4. However, after 36 hours p.i., microcolony-containing IECs for both *Naip1-6^{-/-}* and *Nlrc4^{-/-}* mice were also cleared, indicating that caspase-1 activation is not the only mechanism at play promoting epithelial restriction of *Salmonella*. Finally, the authors demonstrated this microcolony phenotype was reliant on IEC-exclusive Naip-Nlrc4 inflammasome activation through a series of elegant bone marrow transplant studies as well as the use of *Naip1-6*^{ΔIEC-/-} mice.

1.4.4 IEC intrinsic Caspase-11 axis

Our study published in the same issue of Cell Host & Microbe, focused on the role of the noncanonical inflammasome in restricting *Salmonella* burdens (Knodler *et al.*, 2014). We demonstrated that $Casp11^{-/-}$ mice carried higher *Salmonella* loads in their cecal tissues at 7 days p.i., but we observed no significant difference in systemic pathogen loads, which is consistent with previous reports (Knodler *et al.*, 2014, Broz *et al.*, 2012). We also observed the microcolony phenotype in the epithelial cells of $Casp11^{-/-}$ mice, but at 24 hours p.i. (Knodler *et al.*, 2014). Upon staining the ceca of wildtype mice, we found individual intracellular bacteria that were scattered throughout the epithelium. However, $Casp11^{-/-}$ mice displayed numerous IECs containing greater than five bacteria per cell. These microcolonies were observed only rarely in wildtype mice, suggesting that epithelial cell sloughing may be delayed in $Casp11^{-/-}$ mice. This phenotype extended to other organ sites, as infection of the gallbladder epithelium by *Salmonella* was also marked by heavy intracellular infection of the epithelium that remained intact in $Casp11^{-/-}$ mice, whereas infected epithelial cells in wildtype mice were largely shed into the gallbladder lumen.

Caspase-11 (or its human orthologue caspase-4) also governed the activation and secretion of the cytokine IL-18 (Knodler *et al.*, 2014). Infection of an immortalized human intestinal epithelial cell line by *Salmonella* induced robust IL-18 secretion that correlated with inflammasome activation and the presence of caspase-4. This was also observed in a murine oral *Salmonella* infection model, where *Casp11^{-/-}* and *Casp1/11^{-/-}* deficient mice demonstrated impaired *ex vivo* release of IL-18 from intestinal explants as compared to wildtype mice. Conversely, IL-1 β secretion in this infection model was found to be dependent on the presence of caspase-1. The tissue localization of IL-18 and IL-1 β signals differed between the intestinal epithelium and the underlying lamina propria. Both compartments produced cleaved IL-18 after a *Salmonella* infection, however only the lamina propria displayed signal for IL-1 β . This is consistent with other reports detailing that IECs cannot produce IL-1 β protein and this inability is likely controlled at the expression level due to the lack of *il1\beta* transcripts within IECs (Woywodt *et al.*, 1994, Mahida *et al.*, 1989, Youngman *et al.*, 1993, Panja *et al.*, 1995, Daig *et al.*, 2000, Figure 1.2).

1.4.5 Interplay between canonical and non-canonical IEC inflammasomes

Currently a role for the canonical Naip/Nlrc4 and non-canonical caspase-11 inflammasome pathways has been established in driving IEC resistance against Salmonella invasion (Knodler et al., 2014, Sellin et al., 2014). It also appears, based on our own work as well as DSS-induced colitis reports, that an IEC intrinsic Nlrp3 inflammasome exists (Knodler et al., 2014, Oficjalska et al., 2015, Wynosky-Dolfi et al., 2014). However, the contribution of each separate IEC inflammasome to host protection against Salmonella has not yet been established nor has it been elucidated whether each inflammasome operates independently, or in unison, to ultimately clear the infection. Controversy also surrounds the downstream products of inflammatory caspase activation. Caspase-1 and -4/-11 activation ultimately results in IL-1β and IL-18 secretion as well as host cell pyroptosis. There are conflicting reports about which caspase is responsible for which function. Stowe *et al.* proposed that caspase-4/-11 activation alone induces pyroptosis, while others suggest caspase-1 activation also plays a role in pyroptosis (Stowe *et al.*, 2015, Man and Kanneganti, 2015). Some researchers report that caspase-4/-11 induces caspase-1 activation and subsequent processing of IL-1 β and IL-18, while others observe caspase-4/-11 direct processing of IL-18 (Stowe et al., 2015, Storek and Monack, 2015, Knodler et al., 2014, Man and Kanneganti, 2015). Finally, questions still remain concerning the intracellular location of Salmonella required for IEC inflammasome activation. Sellin and colleagues observed their IEC microcolonies remained largely contained within the SCV while it has been established that within BMDM, the intracellular Salmonella must escape the SCV and replicate in the cytosol to be detected by caspase-11 (Sellin et al., 2014, Aachoui et al., 2013, Knodler et al., 2014).

1.4.6 Do IECs undergo pyroptosis?

Eukaryotic cells can undergo several distinct forms of cell death that produce different morphological and physiological outcomes (Bergsbaken *et al.*, 2009). Pyroptosis, or inflammatory cell death, is a relatively new form of cell death and defined as cell death resulting from the inflammasome-dependent processing of Gsdmd (Aglietti *et al.*, 2016, de Vasconcelos *et al.*, 2019, Kayagaki *et al.*, 2015, Shi *et al.*, 2015). These cleaved Gsdmd fragments self-assemble into small pores that intercalate within the plasma membrane, ultimately resulting in cell death through the loss of the cell membrane potential and leakage of cellular contents (Kayagaki *et al.*, 2015, Shi *et al.*, 2015).

The cellular mechanism of pyroptosis are currently under investigation and the majority of studies used to define this pathway utilize macrophages as their model cell type. Thus, it is important to discover if the cell death observed in *Salmonella*-infected IECs is also due to pyroptosis, such as observed in *Salmonella*-infected macrophages. Previous work in IECs has focused on the use of fluorescent dyes to measure cell membrane permeability. Sellin and colleagues described use of a transgenic IEC-expressing cytosolic red-fluorescent protein (dsRed) mouse and conclude that IEC cell lysis occurred after cell expulsion because they observed high red fluorescent activity in intact and actively expelling IECs during an acute *Salmonella* infection (Sellin *et al.*, 2014). The authors only observed reduced dsRed activity in luminally expelled IECs, suggesting these shed cells contained a compromised cellular membrane that permitted fluorescent protein to leak and the cell to dim. However, in a separate study by Rauch and colleagues, enteroids were treated with a recombinant inflammasome activating protein called FlaTox that resulted in rapid IEC uptake of the small fluorescent dye

propidium iodide (PI) and this influx occurred prior to IEC expulsion (Rauch et al., 2017). These conflicting reports of IEC membrane permeability kinetics during inflammasome activation is most likely due to Gsdmd pore size as well as decreasing cellular membrane integrity over the course of IEC lysis. Single cell studies of macrophages have defined that molecules pass through Gsdmd pores over time in accordance to their size, in that a small molecule such as PI (0.668kDa) can enter a pyroptotic macrophage earlier than a larger macromolecule such as dsRed (25.8kDa) can escape (DiPeso *et al.*, 2017). Macrophage pyroptosis dynamics describe Ca^{2+} influx preceding entry of larger DNA intercalating agents, which gain access later in the pyroptotic process as well as loss of small molecules such as ATP which did not require overt cell lysis (de Vasconcelos et al., 2019, Russo et al., 2016). Interestingly, if pyroptotic macrophages are osmotically stabilized through the addition of glycine, loss of large transgenically expressed fluorescent molecules through cellular lysis can be suppressed, however the cell remains permeable to small molecules in a Gsdmd-dependent mechanism (DiPeso et al., 2017). It remains to be seen if the delay in complete plasma membrane integrity loss of dying IECs observed by Sellin et al. is due to increasing Gsdmd pore size over the course of pyroptotic expulsion into the lumen or if there exist osmotically stabilizing forces exerted by the mucosa that maintain infected IEC membrane integrity until it reaches the lumen. Also, there is a recent report that describes inflammasome-mediated restriction of Salmonella intracellular macrophage growth that occurs prior to cell lysis, that is both caspase-1 and caspase-11 dependent, but independent of Gsdmd (Thurston et al., 2016). The identity(s) of the cytosolic antimicrobial agent(s) mediating this protective effect remains unknown as well as its potential contribution to IEC inflammasome-dependent intracellular Salmonella restriction.

Once overt cellular lysis has occurred, it is unclear if *Salmonella* utilizes the luminal cellular debris to launch a secondary infection or if it is trapped in the IEC carcass such as was described with the 'pore-induced intracellular traps' (PITs) produced by infected macrophages (Jorgensen *et al.*, 2016). Although pyroptosis ruptures the macrophage plasma membrane allowing cytosolic contents to escape, Jorgensen *et al.* observed the cellular remnants snare organelles and *Salmonella*, and neutrophils can then detect and engulf PITs through their class A scavenger receptors as well as complement receptors to ultimately clear the infection (Jorgensen *et al.*, 2016).

1.4.7 IEC intrinsic Caspase-8

Recently, Rauch and colleagues extended these studies, confirming that Nlrc4-based pyroptotic expulsion of IEC is an epithelial-intrinsic mechanism that occurs in both the small intestine and cecum (Rauch *et al.*, 2017). Naip-mediated activation of epithelial Nlrc4 resulted in caspase-1 activation followed by processing of Gsdmd. The N-terminal p30 fragments of Gsdmd selfassociate to form a ring shaped oligomer at the cell membrane that destabilizes ionic gradients resulting in water influx, cell swelling and eventual membrane rupture (Aglietti *et al.*, 2016, Ding *et al.*, 2016, DiPeso *et al.*, 2017). Simultaneously, an actin network produced by nearby IEC surrounds the pyroptotic IEC, sealing off the apical surface to maintain epithelial barrier continuity (Rauch *et al.*, 2017, Rosenblatt *et al.*, 2001). Interestingly, these pores appear too small for bacteria as well as most viruses to escape, potentially trapping them within the cytoskeleton of the ruptured cells (Jorgensen *et al.*, 2016). Upon enterocyte inflammasome activation, IL-18 and prostaglandin E2 (Pge2) are released from the dying cell, resulting in subsequent IEC IFN- γ production and neutrophil recruitment as well as the triggering of intraluminal fluid accumulation that helps flush the shed enterocytes away from the site of infection (Rauch *et al.*, 2017, Knodler *et al.*, 2014).

In the absence of Gsdmd or caspase-1 activation, caspase-8 can also induce enterocyte cell death by associating with the Nlrc4 inflammasome via the adaptor protein Asc (Rauch *et al.*, 2017). IEC shed through caspase-8 activation were found to be impermeable to propidium iodide staining, suggesting expelled enterocytes were instead undergoing an apoptotic-like form of cell death instead of pyroptosis. Despite constitutive expression of caspase-8 in epithelial cells at the villus tips, the contribution of this caspase to enterocyte expulsion during Nlrc4 inflammasome formation appeared to be minor when in the presence of caspase 1 (Wittkopf et al., 2013, Rauch et al., 2017). This suggests caspase-1 induced pyroptosis is a more robust response, as caspase-8 only weakly associates with the Nlrc4 inflammasome. Thus, the caspase-1 inflammasome axis potentially downregulates caspase-8 activation or alternatively, secondary signals within the cellular microenvironment are responsible for regulating caspase-8 redundancy. In immune cells, the degree of inflammasome activity relies on various posttranslational modifications, and studies have implicated potential roles for Dectin-1/Syk axis, Pyk2/FAK as well as PKC8. All of these proteins are expressed in enterocytes, however it remains to be determined if they modulate Asc and/or the Nlrc4 activation status in IEC (Hara et al., 2013, Qu et al., 2012, Cohen-Kedar et al., 2014, Wang et al., 2006, Chung et al., 2016, Gao et al., 2015, Yang et al., 2017). Further study is required in the elucidation of the protein network connecting distinct extracellular and cytoplasmic signalling pathways to trigger the extrusion of infected IEC while maintaining the epithelial barrier.

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1.4.8 Inflammasome signalling in intestinal goblet cells

While inflammasome signalling likely occurs within all IEC subsets, its role in enteric defense has only been investigated in enterocytes and goblet cells. The Nlrp6 inflammasome was recently shown to be a major regulator of goblet cell mucin exocytosis. Although the mucin layer of Nlrp6^{-/-} mice appears normal at baseline, these mice demonstrated impaired mucin secretion in response to TLR-antigenic challenge (Birchenough et al., 2016). This is most likely due to the inability of Nlrp6^{-/-} sentinel goblet cells to detect and respond to enteric infection (Birchenough et al., 2016). To date, sentinel goblets cells have only been characterized in the colon. Sentinel goblet cells are a subset of goblet cells that have the ability to detect microbial products that pass through the mucus layer (Birchenough et al., 2016). These specialized goblet cells utilize TLRs to sense and endocytose bacterial products, which triggers the synthesis of Nox/Duox reactive oxygen species, cumulating in Nlrp6 inflammasome activation and release of cytoplasmic Ca²⁺ stores (Birchenough et al., 2016). This triggers a two-fold response in which the sentinel goblet cell secretes its own mucin granules, while simultaneously signalling via intracellular gap junctions to underlying goblet cells to also release their granules. The result is a massive exocytosis of mucins that physically pushes the bacteria away and protects the depths of the intestinal crypt. This is accompanied by the sloughing of the activated sentinel goblet cells along with any remaining microbial products they had endocytosed. The discovery of this subset of goblet cells supports the hypothesis that the makeup of the intestinal epithelium is far more complex than previously anticipated, and that the diversity displayed along the crypt axis is integral for intestinal mucosal defense.

1.5 Salmonella epithelial infection model systems

To elucidate the interaction between *Salmonella* and the epithelial inflammasome, appropriate model systems must be applied. There are numerous *Salmonella* models that facilitate the exploration of its intestinal and systemic pathogenesis (Higginson *et al.*, 2016, Santos *et al.*, 2001, Yin and Zhou, 2018). However, studying *Salmonella*-intestinal epithelial interactions require gastroenteritis models (summarized in Table 1.1) that enable the study of the initial colonization of the intestine by *Salmonella*.

| | Reference | Model description |
|----------|---|---|
| In vitro | (Giannella <i>et al.</i> , 1973b) | HeLa cell line (human cervical adenocarcinoma) |
| | (Gahring <i>et al.</i> , 1990, Finlay and Falkow, 1990) | Caco-2 cell line (human intestinal adenocarcinoma) |
| | (Morales Espinosa <i>et al.</i> , 1993) | HT-29 cell line (human intestinal adenocarcinoma) |
| | (McCormick <i>et al.</i> , 1993) | T84 cell line (human intestinal adenocarcinoma) |
| | (Kusters et al., 1993) | INT-407 (human colorectal carcinoma) |
| | (Nickerson <i>et al.</i> , 2001) | 3D tissue aggregates utilizing INT-407 cell line |
| | (Honer zu Bentrup <i>et al.</i> , 2006) | 3D tissue aggregates utilizing HT-29 cell line |
| | (Martinez-Argudo and Jepson, 2008) | M cell model (co-culture of polarized Caco-2 with Raji B cells) |
| | (Gagnon et al., 2013) | HT-29 MTX cell line (mucus secreting HT-29) |
| | (Dostal et al., 2014) | Effluents from colonic fermentation system inoculated with child gut microbiota combined with Caco-2/HT-29-MTX monolayers |
| | (Zhang et al., 2014b) | Murine enteroids (derived from intestinal crypts) |
| | (Forbester <i>et al.</i> , 2015) | Human enteroids (derived from human induced pluripotent stem cells) |

Table 1.1 Salmonella Typhimurium gastroenteritis models.

| | (Co et al., 2019) | 'Apical-out' human enteroids |
|---------|-----------------------------------|---|
| | This work | Murine enteroid monolayers |
| Ex vivo | (Frost et al., 1997) | Calf ileal loop model |
| | (Haque <i>et al.</i> , 2004) | Human intestinal <i>in vitro</i> organ culture (IVOC; immobilized ileal biopsies) |
| | (Boyle et al., 2016) | Perfusion of the rat small intestine |
| oviv nl | (Takeuchi, 1967) | Opium pretreated guinea pig |
| | (Giannella <i>et al.</i> , 1973a) | Rabbit (ileal loop) |
| | (Barthel et al., 2003) | Streptomycin pretreated C57BL/6 mice |
| | (Robertson <i>et al.</i> , 2003) | Young rat |
| | (Tsolis et al., 1999) | Calf |
| | (Zhang <i>et al.</i> , 2014a) | Neonate mice |

1.5.1 Gastroenteritis animal models of Salmonella

There are a variety of small and large animal models of *Salmonella* gastroenteritis that each present with their own unique features of the bacterium's pathogenesis. However, large animals such as cows and calves require specialized housing requirements that are not easily accessible to the majority of research facilities, while ileal loop models require a high level of surgical expertise. Therefore, the streptomycin pretreatment mouse model is the most commonly employed due to inbred murine genetic homogeneity, the array of genetic deficient mice available and finally, overall cost efficiency in terms of housing as well as the number of animals required to observe biological significance.

1.5.1.1 Streptomycin pretreatment mouse model of *S*. Typhimurium infection

To model gastroenteritis in mice, the animals are first pretreated orally with streptomycin to transiently disrupt the microbiota (approximately >10-fold reduction in density and composition), thereby reducing colonization resistance and opening a niche for Salmonella to colonize the cecum and colon (Kaiser et al., 2012). Here, Salmonella invades the intestinal cecal mucosa and can be observed inside IECs as early as 6h post infection (p.i.) (Sellin et al., 2014). From 12-72h p.i., Salmonella establishes its intestinal niche, causing localized inflammation and spreading systemically (Barthel et al., 2003, Sellin et al., 2014). This is demonstrated by the worsening cecal pathology, characterized by epithelial hyperplasia, mucosal edema and infiltration of the mucosa by polymorphonuclear leukocytes as well as monocytic phagocytes (Barthel et al., 2003). The epithelium is also badly damaged with widespread cell sloughing and loss of the mucin-filled goblet cells, and at 72h p.i. especially in susceptible genetic deficient mice, the loss of regions of epithelium can be observed (Barthel et al., 2003, Kaiser et al., 2012). Some drawbacks to this model include its inability to mimic the diarrheal symptoms typically observed in human salmonellosis, since only minimal water content increases are observed in stools during infection (Higginson et al., 2016). Also, since an antibiotic is used to reduce colonization resistance, this model cannot be used to study the interaction of Salmonella with the microbiota to discover which microbial relationships impact pathogenesis. However, it is an excellent tool for exploring Salmonella intestinal colonization and mucosal inflammation in vivo and has been a cornerstone in uncovering the mechanisms behind *Salmonella* pathogenesis.

1.5.2 Cell culture models of Salmonella

Epithelial cell culture lines are also heavily employed to study *Salmonella* pathogenesis (Table 1.1). This is a cost-effective, robust, simple to maintain and genetically manipulatable model that has uncovered a number of mechanisms by which *Salmonella* interacts with and manipulates host cell signaling pathways. However, immortalized cell lines lack the cellular subtype complexity of the intestinal epithelium and due to their carcinoma origin, exhibit differential signaling pathways compared to primary cells, such as those regulating cell death (Finlay and Brumell, 2000). This suggests novel *Salmonella* colonization and invasion mechanisms could be potentially overlooked due to the models typically applied.

In 2009, a novel three-dimensional (3D) *in vitro* tissue model was described by Dr. Hans Clevers' group termed 'enteroids' (Sato *et al.*, 2009, Sato *et al.*, 2011). These spheroid "gut balls" maintain many of the physiological relevant features of the *in vivo* tissue including a polarized epithelium, crypt spatial recapitulation as well as the inclusion of rarer secretory IEC subtypes. To isolate these cells, the entire crypt or the stem cells themselves are extracted from intestinal tissue, or alternatively, derived from embryonic or induced pluripotent stem cells, and then the cells are embedded in Matrigel domes (a gelatinous extracellular matrix protein mixture secreted by Engelbreth-Holm-Swarm mouse sarcoma) (Forbester *et al.*, 2015, Forbester *et al.*, 2018, Sato *et al.*, 2011). The enteroids are supplemented with media containing recombinant Wnt3a, R-spondin-1 and Noggin which aid in the proliferation of the intestinal Lgr5+ stem cells as well as general growth and anti-apoptotic factors (Sato *et al.*, 2011). The spheroids grow and differentiate, forming crypts buds that extend from the central lumen presenting with a "starfishlike" gross morphology and producing a 3D enclosed epithelial model.

1.5.2.1 Salmonella enteroid infection model

Recently, enteroids have been applied to the study of infectious disease including *Salmonella* pathogenesis. The first instance of enteroids as a *Salmonella* infection model was reported by Zhang and colleagues (Zhang *et al.*, 2014b). Here, enteroids were removed from the Matrigel and gently resuspended in media containing *Salmonella* for 30 mins. *Salmonella* infected the enteroids by accessing the basolateral membrane, thereby inducing gross morphological changes through disruption of epithelial tight junctions (Zhang *et al.*, 2014b). The authors also detected activation of the NF- $\kappa\beta$ pathway as well as increased transcription of inflammatory cytokines IL-2, IL-4, IL-6, TNF α and IFN γ , mirroring the observations of epithelial inflammatory responses to infection seen *in vivo* and in other *in vitro* studies (Zhang *et al.*, 2014b).

Expanding on this model, Co *et al.* developed a technique to reverse enteroid cellular polarity to expose the apical surface, (which under conventional Matrigel culturing is contained within the spheroid) and infect these 'flipped' enteroids with *Salmonella* (Co *et al.*, 2019). Inspired by culturing of Madin-Darby canine kidney spheroids, the author first grew enteroids in Matrigel and once the spheroids had reached an adequate size, then removed the extracellular matrix and transferred the enteroids into a suspension culture. This induced a shift in the cellular polarity of the spheroid, inducing the apical cellular surface to flip outwards and the basolateral membrane to internalize and face the inner spheroid lumen. These 'apical-out' enteroids maintained cellular polarity for at least three days and displayed overt barrier function, as well as IEC secretory cell differentiation and polarized absorption of nutrients (Co *et al.*, 2019). The exposure of the apical surface dramatically increased the incidence of *Salmonella* intracellular infection compared to
conventionally cultured enteroids and the authors observed preferential apical membrane infection of 'half-flipped' mixed polarity enteroids. Apical-out enteroids also demonstrated intracellular *Salmonella* infection-induced cell shedding that has been observed *in vitro* in Caco-2 cells as well as *in vivo* in the murine intestine (Knodler *et al.*, 2014, Knodler *et al.*, 2010, Sellin *et al.*, 2014).

The recent development of enteroids as a primary intestinal epithelial cell infection model is a major advancement in *Salmonella*-IEC pathogen-host interactions and will facilitate new avenues of study in *Salmonella* infection dynamics. However, enteroids, like traditional cell culture, can only explore biological phenomenon intrinsic to the intestinal epithelial cells themselves. This is in contrast to the *in vivo* intestinal epithelium which is in constant contact with signals from its surroundings, including the luminal microbiota and underlying immune and mesenchymal cells (Allaire *et al.*, 2018). Here the epithelium integrates these extrinsic signals into differing protein expression levels to produce a cumulative mucosal response across all tissue levels. Therefore, further study of *Salmonella* enteroid infections should incorporate mucosal signaling molecules appropriate to the stage of infection to better model an intestinal mucosal infection.

1.6 Thesis objectives

The overall objective of this thesis is to identify and characterize the effect of the inflammasome on the IECs in response to *Salmonella* infection. I hypothesize that the intestinal epithelium utilizes inflammasome signaling to coordinate multiple layers of innate defense at the gut mucosal surface to ultimately restrict enteric pathogen infections and systemic spread. The overall working hypothesis will be tested through the following specific aims (summarized Fig 1.4):

1: Uncover the function of caspase-11 in IEC innate defense.

2: Determine the individual contributions of caspase-1 and -11 to IEC and host innate protection from *Salmonella*.

3: Examine the role of downstream IEC-driven antimicrobial responses triggered by inflammasome activation and their role in the promotion of host defense.

Together, these objectives will define the role the IEC inflammasome plays in the protective functions of the intestine, as well as its contribution to overall host enteric defense.



Figure 1.4 Intestinal mucosa innate defense against Salmonella by the inflammatory caspases.

Outstanding questions concern 1) Do IECs utilize caspase-11/4 to protect against intracellular *Salmonella*? 2) What are the contributions of caspase-1 and -11 in IEC innate defense? 3) What role does the inflammasome play in overall mucosal antimicrobial defense against *Salmonella*?

Chapter 2: Non-canonical inflammasome activation of caspase-4/caspase-11 mediates epithelial defenses against enteric bacterial pathogens

2.1 Introduction

Inflammasomes mediate inflammatory host defenses, including pyroptosis, a specialized form of cell death, and the cleavage and activation of the proinflammatory cytokines, IL-1β and IL-18 (Ng and Monack, 2013). These responses require the actions of inflammatory caspases, specifically caspase-1, -4, -5, and -12 in humans and caspase-1, -11, and -12 in mice (Ng and Monack, 2013). Caspase-1, the best characterized to date, is cleaved and activated upon recruitment to a multiprotein complex, the inflammasome. Inflammasome assembly is triggered by cytosolic Nod-like receptors (NLRs) that sense microbial- or danger-associated molecular patterns (DAMPs) (Lamkanfi, 2011). Aside from caspase-1-containing inflammasomes, a "noncanonical" inflammasome has been recently described in mouse macrophages that respond to intracellular bacterial lipopolysaccharide (LPS) (Kayagaki *et al.*, 2011). Caspase-11 is activated independently of the LPS receptor, Toll-like receptor 4 (TLR4); the cytosolic sensor for LPS is unknown (Hagar *et al.*, 2013, Kayagaki *et al.*, 2013). It also remains unclear whether human caspase-4 and/or -5 represent functional orthologs of murine caspase-11.

The primary effectors of inflammasome-mediated control of bacterial infections are believed to be immune cells, such as monocytes, macrophages, and dendritic cells. Because intestinal epithelial cells (IECs) lie at the host-microbial interface and are an important source of proinflammatory cytokines, we hypothesized that inflammasome activation in these cells plays a previously unrecognized role in responding to bacterial infections of the gut. The Gramnegative bacterium *Salmonella enterica* serovar Typhimurium (*S*. Typhimurium) infects the IECs of several mammalian species, including humans, causing gastroenteritis (Santos *et al.*, 2001). We previously showed that *S*. Typhimurium-infected IECs undergo pyroptosis and release IL-18 (Knodler *et al.*, 2010). Using *S*. Typhimurium as a model enteric pathogen, we herein describe how the noncanonical epithelial inflammasome promotes host defense and gut inflammation in response to enteric bacteria.

2.2 Results

2.2.1 Intestinal epithelial cells require caspase-4 activity for IL-18 secretion

To determine if *S*. Typhimurium-induced IL-18 secretion required one or more inflammatory caspases, we tested a panel of irreversible, cell-permeable caspase inhibitors. Polarized human colonic epithelial cells (C2Bbe1) were infected with *S*. Typhimurium and secretion of two proinflammatory cytokines, IL-18 and IL-8, assayed by ELISA (Knodler *et al.*, 2010, Jung *et al.*, 1995). IL-18 requires proteolytic processing by an inflammatory caspase prior to secretion, whereas IL-8 does not (van de Veerdonk *et al.*, 2011). Caspase-1, -4, and -5 inhibitors significantly reduced *S*. *Typhimurium*-induced IL-18 secretion (Figure 2.1A), implicating inflammatory caspase catalytic activity. None of the inhibitors affected IL-8 secretion (Figure 2.1A).



Figure 2.1 Inhibition and knockdown of caspase-4 affects IL-18, but not IL-8, secretion from intestinal epithelial cells.

Polarized human colonic epithelial cells (C2Bbe1) were infected with *S*. Typhimurium and cell culture supernatants collected at 10h p.i. for cytokine analysis of the cytokines IL-18 or IL-8. A. Cells were incubated with an array of fluoromethyl ketone (FMK) caspase peptide inhibitors (20 μ M) from 30min - 10h p.i. B. Cells were treated with small interfering RNA (siRNA) to deplete *CASP1*, *CASP4*, *CASP5* or *IL18*. Results are shown as the mean \pm SD from n≥3 independent experiments. Asterisks indicate data statistically different from untreated infected samples (p<0.05, ANOVA with Dunnett post-hoc test). C. List of FMK caspase peptide inhibitors and their targets used in the experiments presented in Figure 2.1A.

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IL-18 is synthesized as a 23 kDa inactive precursor that requires cleavage by an active

inflammatory caspase to obtain its mature 18 kDa form (van de Veerdonk et al., 2011). By

immunoblotting, mature IL-18 was secreted upon S. Typhimurium infection of IECs

(Figure 2.4C). siRNA-mediated knockdown of caspase-4, but not caspase-1 or -5, prevented the

processing and release of mature IL-18. S. Typhimurium infection also led to the time-dependent

release of IL-18 from HCT 116 cells (Figure 2.4D), a colonic epithelial cell line that expresses

only inflammatory caspase-4 (Figure 2.2), confirming that IL-18 secretion does not require

caspase-1 in human IECs.



Figure 2.2 Caspase-1, caspase-4 and caspase-5 expression in human epithelial cells.

A. Whole cell lysates from the indicated human epithelial cell lines and PMA activated THP-1 monocytes were subject to immunoblotting with antibodies against caspase-1 (pro-form: 46kDa), caspase-4 (pro-form: 43kDa), IL-18 (pro-form: 24kDa), IL-1 β (pro-form: 33kDa) and actin. Caco-2 C2Bbe1, colorectal adenocarcinoma; HCT-8, ileocecal colorectal adenocarcinoma; HCT 116, colorectal carcinoma; HeLa, cervical adenocarcinoma; HT-29, colorectal adenocarcinoma; HUT 80, duodenal adenocarcinoma; SW480, colorectal adenocarcinoma. Long exp (exposure) was ~5 times the duration as the short exp B. qPCR analysis of caspase-1, caspase-4 and capase-5 mRNA expression in human epithelial cell lines (light grey bars), primary human epithelial cells (dark grey bars) and PMA-activated THP-1 monocytes (white bars). Primary cells are colonic epithelial cells (21 week fetus) and small intestinal epithelial cells (19 week fetus). Total RNA was DNase-treated and reverse transcribed to cDNA. Expression of each caspase was analyzed using qPCR and normalized to ribosomal phosphoprotein P0 (RPLP0). ND, not detected. For cell lines, results are means \pm SD from three independent RNA preparations. Printed with permission from Cell Host & Microbe. 2014 Aug 13;16(2):249-256.





Caco-2 C2Bbe1 cells were electroporated with siRNA directed against caspase-1 (CASP1), caspase-4 (CASP4), caspase-5 (CASP5), interleukin-18 (IL-18) or a nontargeting control (NT) and seeded on collagen-coated wells. A. RNA was isolated 48h post-electroporation, DNase-treated and reverse transcribed to cDNA. qPCR was used to quantify mRNA for caspase-1, caspase-4, caspase-5, IL-18 and the reference gene, RPLP0. Relative gene expression compared to the NT control was calculated using a $\Delta\Delta$ Cq method. Dashed line at 1.0 indicates no change in gene expression. Results are means ± SD from three independent experiments. Statistics were not conducted on this data set because they were generated solely to demonstrate that the siRNA knockdown was successful for Fig 2.4 B. Immunoblots of whole cell lysates from siRNA-treated Caco-2 C2Bbe1 cells at 48h post-electroporation for caspase-1 (pro-form: 46kDa), -4 (pro-form: 43kDa) and IL-18 (pro-form: 24kDa). No commercially available anticaspase-5 antibody was found to be specific for caspase-5 detection. Actin serves as a loading control. Results are representative of at least three independent experiments. Printed with permission from Cell Host & Microbe. 2014 Aug 13;16(2):249-256.



Figure 2.4 Caspase-4 is required for IL-18 secretion and processing in human intestinal epithelial cells. A. C2Bbe1 cells were electroporated with siRNA-targeting caspase-1 (CASP1), caspase-4 (CASP4), caspase-5 (CASP5), interleukin-18 (IL-18), or a nontargeting control (NT). Polarized monolayers were mock-infected or infected with S. Typhimurium or enteropathogenic E. coli (EPEC). Apical and basolateral culture supernatants were collected at 10 h p.i. from the top or bottom transwell receptacles and secreted IL-18 determined by ELISA. *p < 0.05, significantly different from infected, NT siRNA conditions. B. C2Bbe1 cells were incubated in the presence of 1 µg S. Typhimurium LPS or nucleofected with a dilution series of LPS. At 16 h posttreatment, cell culture supernatants were assayed for IL-18 (gray bars) and IL-8 (white bars) by ELISA. *p < 0.05, significantly different from nucleofection with water. C. Immunoblots of whole-cell lysates (WCL) and supernatants (SN) probed for actin and IL-18 (pro- (pro-form): 24kDa; mature: 18kDa). HeLa cells were transfected with the indicated siRNA and infected 48 h later with S. Typhimurium. Samples were collected at 10 h pi. Representative of three independent experiments. D. HCT 116 cells were mock infected or infected with S. Typhimurium, and cell-culture supernatants were assayed for IL-18 by ELISA. E. HeLa cells were treated with NT or caspase-4 siRNA and mock infected or infected with mCherry S. Typhimurium. Whole-cell lysates were analyzed by immunoblotting with antibodies against caspase-4 (pro-form; 43kDa) and actin (representative of three experiments). IL-18 in culture supernatants was assayed by ELISA (black bars). Caspase activity was measured after incubation with FAM-YVAD-FMK FLICA reagent. The number of cells with active caspase-1/4/5 was assessed by fluorescence microscopy (gray bars). Asterisks indicate significantly different data. (A), (B), (D), and (E) show mean ± SD. See also Figures 2.1–2.3. Printed with permission from Cell Host & Microbe. 2014 Aug 13;16(2):249-256.

In mouse macrophages, cytosolic Gram-negative bacteria activate the caspase-11 inflammasome, via intracellular LPS detection (Aachoui *et al.*, 2013, Hagar *et al.*, 2013, Kayagaki *et al.*, 2013). LPS delivered to the cytosol of human IECs triggered the caspase-4 inflammasome; extracellular LPS did not (Figure 2.4B). Cytosolic LPS stimulated IL-18, but not IL-8, release in a dose-dependent manner (Figure 2.4B), implying that IECs possess a cytosolic LPS sensing pathway. Unexpectedly, infection with extracellular enteropathogenic *Escherichia coli* (EPEC) also led to a caspase-4-dependent induction of IL-18 release from colonic epithelial cells (Figure 2.4A).

Expression of some inflammatory caspases is transcriptionally regulated (Broz et al., 2012,

Kayagaki et al., 2011, Lin et al., 2000). However, caspase-4 is highly expressed in human IECs

(Figure 2.2) and only modestly induced upon infection (Figure 2.4E), indicating that its

expression does not require inflammatory stimulation. The peak times for caspase-1/4/5 activity

and IL-18 secretion were concurrent in infected epithelial cells (Figure 2.4E). Moreover, siRNA

knockdown demonstrated that almost all secreted IL-18 and the majority of FAM-YVAD-FMKpositive cells at 20 h post-infection (p.i.) was due to caspase-4 activity (Figure 2.4E). In support of recent findings, we conclude that enteric bacterial infection of human IECs progressively activates caspase-4, resulting in IL-18 processing and secretion (Kobayashi *et al.*, 2013).

2.2.2 Caspase-11 is required for IL-18 secretion during gut inflammation

To test the *in vivo* relevance of the epithelial inflammasome in proinflammatory cytokine release, we used the *S. Typhimurium*-induced mouse model of gastroenteritis (Barthel *et al.*, 2003, Miller *et al.*, 1956). Caspase-11 is the murine ortholog of human caspase-4 and -5 (Ng and Monack, 2013).IL-18 and IL-1 β secretion from infected cecal tissues of wildtype C57BL/6, *Casp11^{-/-}*, or *Casp1^{-/-} Casp11^{-/-}* mice was quantified (Figure 2.5A). Secreted IL-18 levels were significantly lower in cecal explants from *Casp11^{-/-}* mice compared to wildtype mice. IL-1 β release was also detectable, consistent with the presence of various myeloid cells in cecal tissues, but there was no difference between wildtype and *Casp11^{-/-}* mice (Figure 2.5A). By contrast, both IL-18 and IL-1 β release from *Casp1^{-/-} Casp11^{-/-}* mouse cecal tissues were significantly reduced compared to wildtype mice. Hence, caspase-11 is the predominant inflammatory caspase controlling IL-18 release during intestinal *S.* Typhimurium infection, whereas caspase-1, rather than caspase-11, governs intestinal IL-1 β responses.

Pro-IL-1 β and pro-IL-18 processing upon pathogen activation of the caspase-11 inflammasome in mouse macrophages requires the Nod-like receptor family member, NLRP3, and the adaptor apoptosis-associated speck-like protein (ASC/PYCARD) (Kayagaki *et al.*, 2011). In ASC- and NLRP3-deficient mice, IL-18 and IL-1 β release were both significantly reduced from cecal explants compared to wildtype mice (Figure 2.5A), implicating ASC and NLRP3 in the release of these proinflammatory cytokines during *S. Typhimurium*-induced gastroenteritis.



Figure 2.5 Caspase-11 is required for IL-18, but not for IL-1β, secretion during gut inflammation. A. Streptomycin-pretreated C57BL/6, $Casp11^{-/-}$, $Casp1^{-/-}$ $Casp11^{-/-}$, $Asc^{-/-}$, and $Nlrp3^{-/-}$ mice were orally infected with $\Delta aroA S$. Typhimurium (3 × 10⁶ CFU) and cecal tissues collected at 3 days pi. Tissues were washed and incubated in DMEM for 6 h; culture supernatants were collected; and cytokine levels were measured by ELISA. Each symbol represents one animal. Median is indicated. Results are from ≥2 independent experiments. *p < 0.05; ns, not significant. B. Streptomycin-pretreated C57BL/6 mice were orally infected as in (A), and cecal tissues were collected at 3 days pi. The lamina propria was separated from crypts to enrich for mononuclear and intestinal epithelial cells, respectively. Crypts were further incubated in DMEM for 3 h, and culture supernatants were collected. Protein extracts were analyzed by immunoblotting for pro- and mature forms of caspase-1 (pro-form: 46kDa; mature: 33, 20 and 10kDa), -11 (pro-form: 43kDa; mature: 32 and 10kDa), IL-18 (pro-form: 24kDa; mature: 18kDa), and IL-1β (pro-form: 33kDa; mature: 17kDa). Cytokeratin 19 (CK19) is a marker of epithelial cells, and actin is a loading control. Lysates from two representative mice are shown. Printed with permission from Cell Host & Microbe. 2014 Aug 13;16(2):249-256.

To determine the cellular origin of the secreted IL-1 β and IL-18, whole crypts, enriched for IECs, were separated from the underlying lamina propria of cecal tissues from infected wildtype mice. Higher levels of mature caspase-11 and IL-18 were present in the crypt fraction compared

to the lamina propria. Mature IL-18, but not IL-1 β , was also detected in crypt supernatants (Figure 2.5B). By contrast, expression of mature IL-1 β and caspase-1 was comparatively increased in the lamina propria (Figure 2.5B). Therefore IL-18 activation and secretion primarily occurs in IECs in infected cecal tissues, correlating with mature caspase-11 localization. However, IL-1 β production and activation during *S. Typhimurium*-induced intestinal inflammation is predominantly associated with caspase-1 expression and processing in cells of the lamina propria.

2.2.3 Caspase-4 governs intestinal epithelial shedding rates

S. Typhimurium occupies two distinct niches within human epithelial cells (Knodler *et al.*, 2010, Malik-Kale *et al.*, 2012). Epithelial cells containing cytosolic bacteria die by pyroptosis, ultimately being shed from the monolayer (Knodler *et al.*, 2010). Does caspase-4 promote pyroptotic death of infected IECs? Caspase-4 depletion significantly increased the number of recoverable bacteria in polarized monolayers at 10 h pi, but caspase-1 and -5 had no effect (Figure 2.6B). Conversely, ectopic expression of caspase-4, but not caspase-1 or -5, restricted *S. Typhimurium* growth (Figure 2.6A). Caspase-4 depletion did not affect recoverable bacteria \leq 7 h p.i. (Figure 2.6C), indicating no effect on bacterial internalization or early vacuolar trafficking events. Vacuolar replication of *S. Typhimurium* was unperturbed by caspase-4 knockdown at 10 h p.i. (Figure 2.6D, defined as <40 bacteria/cell; Malik-Kale *et al.*, 2012), whereas cytosolic replication was enhanced (\geq 100 bacteria/cell; Knodler *et al.*, 2014, Malik-Kale *et al.*, 2012) (Figure 2.6D). The increased bacterial burden upon caspase-4 depletion reflects an increased number of IECs containing cytosolic *S.* Typhimurium, which is independent of mature IL-18 release (Figures 2.4B and 2.5B), implicating pyroptosis instead. Most dying cells contained cytosolic *S*. Typhimurium (\geq 100 bacteria/cell) (Figure 2.6E) and had a compromised plasma membrane (Figure 2.6F), regardless of siRNA treatment. However, caspase-4 depletion significantly reduced the proportion of infected, extruding cells with active caspase-1/4/5, i.e., dying by pyroptosis (Figure 2.6F). Furthermore, the frequency at which infected cells were shed from epithelial monolayers was decreased in caspase-4-depleted cells (Figure 2.6G). Therefore *S*. Typhimurium induces IEC lysis by more than one mechanism, one of which is caspase-4 dependent and constitutes a key antimicrobial response by IECs to *S*. Typhimurium infection.



Figure 2.6 Caspase-4 limits bacterial burdens via epithelial cell shedding.

A. C2Bbe1 cells were nucleofected with pCMV6-XL5 (empty vector control), pCASP1, pCASP4, or pCASP5, infected with S. Typhimurium, and solubilized at 8 h p.i. for enumeration of CFU. Mean \pm SD. B. C2Bbe1 cells were treated with siRNA, polarized on semipermeable supports, and infected with S. Typhimurium. CFU were enumerated at 10 h p.i. Mean \pm SD. *p < 0.01 C. C2Bbe1 cells were treated with siRNA, polarized on semipermeable supports, and infected with S. Typhimurium. CFU were enumerated over a time course of infection Mean \pm SD. p < 0.02. D. C2Bbe1 cells were treated as in (B) with siRNA (NT (non-targeting; scrambled) or CASP4) and infected with mCherry S. Typhimurium. Monolayers were fixed at 10 h p.i., immunostained with anti-ZO-1 antibodies, and stained with Hoechst 33342 to label epithelial cell nuclei. The number of bacteria in each infected cell was scored by fluorescence microscopy. Each dot represents one infected cell. Data are representative of at least three experiments. Percentages indicate the number of infected cells containing ≥ 100 bacteria/cell (mean \pm SD, n = 3 experiments). * p < 0.05. C2Bbe1 cells were treated as in (B) with siRNA (NT or CASP4) and infected with S. Typhimurium glmS::mCherry or S. Typhimurium glmS::gfpmut3 for 10 hr. E. were fixed and stained with Hoechst 33342. The number of bacteria in extruding/extruded epithelial cells was scored by fluorescence microscopy. Data were binned into three categories: cells containing 1–19, 20–99, and \geq 100 bacteria. Mean \pm SD. F. Monolayers were incubated with Hoechst 33342 and SYTOX Orange or FAM-YVAD-FMK FLICA. The number of infected, extruding/extruded cells that were SYTOX Orange positive or FLICA positive was scored by fluorescence microscopy. Mean \pm SD *p < 0.01. G. C2BBe1 cells were treated and infected as in (D.) with siRNA (NT or CASP4). At 9 h p.i., monolayers were fixed and immunostained as in (D.). The number of extruding/extruded infected cells was scored by fluorescence microscopy. Mean \pm SD. *p < 0.01. Printed with permission from Cell Host & Microbe. 2014 Aug 13;16(2):249-256.

2.2.4 Caspase-11 restricts bacterial burdens in the intestine

Given that IECs have a noncanonical inflammasome, we assessed the in vivo role of caspase-11

in promoting intestinal host defense against enteric bacteria. Bacterial loads in spleen, liver, and

mesenteric lymph nodes were comparable between wildtype and $Casp11^{-/-}$ mice (Figure 2.7A), in agreement with a nonessential role for caspase-11 in controlling *S*. Typhimurium at systemic sites (Broz *et al.*, 2012). However, $Casp11^{-/-}$ mice had significantly higher pathogen loads in their cecal tissues and lumen (Figure 2.7A) and showed a significant reduction in histopathological features of cecal inflammation (Figure 2.7B). At early time points, initial colonization of cecal tissues by *S*. Typhimurium was dramatically different between wildtype and *Casp11^{-/-}* mice (Figures 2.7C and 2.7D), specifically in epithelial cells (Figure 2.7E and 2.7F). Whereas individual bacteria were scattered throughout the cecal epithelium and lamina propria of both mouse strains, numerous crypt epithelial cells containing clusters of >5 *S*. Typhimurium per cell were evident in *Casp11^{-/-}* mouse (11.8 epithelial cells/ten high-power fields). This colonization phenotype was rarely seen in wildtype mice (1.4 epithelial cells/ten high-power fields, p < 0.01), suggesting that epithelial cell sloughing may be delayed in *Casp11^{-/-}* mice.



Figure 2.7 Caspase-11 limits S. Typhimurium burdens in the gut.

A. Streptomycin-pretreated C57BL/6 and Casp11^{-/-} mice were orally infected with $\Delta aroA S$. Typhimurium (3 × 10⁶ CFU) and bacterial loads in organs and tissues determined at 7 days pi. Data were combined from three independent experiments. Each symbol represents one animal (n = 13 for BL/6, n = 11 for Casp11^{-/-}). The median is indicated. *p < 0.05. B. C57BL/6 wildtype and Casp11^{-/-} mice were infected as in (A). Semiguantitative scoring of inflammation was assessed from hematoxylin and eosin-stained cecum sections as described in the Experimental Procedures. Each symbol represents one animal (scoring range = 0-17). n = 6-16 mice per group. The median is indicated. *p < 0.01. C.-F. Streptomycin-pretreated C57BL/6 and Casp11^{-/-} mice were orally infected with GFPexpressing wildtype S. Typhimurium (10⁶ CFU) (green). To identify the localization of intracellular S. Typhimurium, immunostaining was performed on cecal tissues (day 1 p.i.) using different markers to identify and outline intestinal epithelial cells. Tissue were stained with phalloidin to detect actin (red; C and D), anti-cytokeratin 19 (CK19) (E), or anti-epithelial cell-adhesion molecule (EpCAM) (F) to detect epithelial cells (red; E and F) and DAPI to detect DNA (blue; C-F). Arrows and arrowheads indicate individual and clusters of bacteria, respectively. Scale bars, 50 µm. G. and H. C57BL/6 and Casp11^{-/-} mice were infected intravenously with wildtype GFP-Salmonella (5 \times 10² CFU). Gallbladders were collected at day 4 pi. and stained for CK19 to detect epithelial cells (red) and DAPI to detect DNA (blue). Scale bars, 100 µm. Printed with permission from Cell Host & Microbe. 2014 Aug 13;16(2):249-256.

The mouse gallbladder is another site of epithelial cell colonization by *S*. Typhimurium (Gonzalez-Escobedo and Gunn, 2013, Menendez *et al.*, 2009). Despite no overt difference in the proportion of mice showing evidence of gallbladder infection (8/26 wildtype mice, 7/29 $Casp11^{-/-}$ mice), wildtype and $Casp11^{-/-}$ mice showed histopathological differences (Figure 2.8). Gallbladders of wildtype mice showed extensive shedding of epithelial cells laden with bacteria (Figure 2.7G) and a heavy infiltration of neutrophils (Figure 2.8). Gallbladder epithelial cells of $Casp11^{-/-}$ mice were also filled with *S*. *Typhimurium*, but relatively few had sloughed into the lumen (Figure 2.7H), and there was little evidence of neutrophil infiltration (Figure 2.8). Hence, caspase-11-induced epithelial shedding is important for the clearance of enteric bacteria at mucosal sites *in vivo*.



Figure 2.8 Caspase-11 deficiency delays sloughing of gallbladder epithelial cells during S. Typhimurium infection.

Wildtype (C57BL/6) and *Casp11^{-/-}* mice were either uninfected, or infected i.v. with 500 CFU of wildtype SL1344 *S*. Typhimurium. At day 4 p.i., gallbladder tissues were collected, fixed and stained with hematoxylin and eosin (H&E). Representative images were taken using a Zeiss AxioImager microscope. A. Uninfected panels show no overt histological differences between wildtype and *Casp11^{-/-}* mice. Images of infected tissues represent the range of pathology seen in each mouse strain, with pathology increasing as the panels go left to right. Wildtype infected mice displayed extensive epithelial cell sloughing into the gallbladder lumen, whereas *Casp11^{-/-}* infected mice showed only modest pathology and occasional epithelial cell sloughing. Arrowheads indicate sloughed epithelial cells. Scale bars are 500 µm. B. Infected wildtype mice also demonstrated significant neutrophil infiltration. Arrowheads indicate sloughed epithelial cells, while arrows detail neutrophil infiltration. Scale bars are 100 µm. Printed with permission from Cell Host & Microbe. 2014 Aug 13;16(2):249-256.

2.3 Discussion

In the gastrointestinal tract, a single layer of columnar epithelial cells separates the nonsterile

lumen from the sterile underlying tissues. Historically, these IECs have been primarily regarded

as a mechanical barrier against invading pathogens, whereas the underlying lamina

propria and lymphoid tissues, rich in professional immune cells, are considered the main

immunological responders in the gut to pathogenic challenge (Artis, 2008). However, IECs can

distinguish between commensal and pathogenic microbes and respond accordingly through the release of antimicrobial factors, suggesting they can participate in the regulation of intestinal immune homeostasis. Here, we have demonstrated that noncanonical caspase-4 and caspase-11 inflammasomes govern pathogen clearance and inflammation in vitro and in vivo, respectively, invoking a key role for IECs in gut innate immune defense against enteric bacteria. At present, demonstration of a role for caspase-11 in restricting bacterial pathogen growth in vivo is limited; Casp11^{-/-} mice carry higher loads of Legionella pneumophila in their lungs compared to wildtype mice and succumb to Burkholderia pseudomallei and Burkholderia thailandensis infection, whereas wildtype mice do not (Akhter et al., 2012, Aachoui et al., 2013). In both cases, the mechanisms underlying this control are unknown. Here, we uncovered a mechanism for noncanonical inflammasome-mediated restriction of pathogen growth in vivo. Delayed shedding of infected epithelial cells undergoing pyroptosis explains Casp11^{-/-} mice carrying higher intestinal S. Typhimurium burdens. Epithelial cell extrusion is important for maintaining gut homeostasis and barrier function, and accelerated IEC turnover is a hallmark of infection with many enteric pathogens (Gu and Rosenblatt, 2012) (Laughlin et al., 2014, Wallis et al., 1986, Ritchie et al., 2012, Kang et al., 2004). Noncanonical inflammasome-mediated epithelial cell extrusion may reflect a generalized gut defense mechanism to eliminate infected cells. In support of this concept, we and others have found that S. Typhimurium and EPEC both activate the caspase-4 inflammasome in human IECs (Kobayashi et al., 2013). Moreover, it was recently shown that S. flexneri antagonizes IEC death via the actions of a type III effector, OspC3, which binds to cleaved caspase-4, thereby preventing its activation, inhibiting IL-18 release, and delaying epithelial cell death (Kobayashi et al., 2013). OspC3 does not bind caspase-5 or -11 and the *in vivo* relevance of caspase-4 inhibition by *S. flexneri* remains unknown.

The extent to which human caspase-4 and/or -5 are functional orthologs of mouse caspase-11 remains unclear. Tissue expression of caspase-4 is much more widespread than is caspase-5, suggesting cell-type-specific or site-specific roles in inflammasome activation and inflammatory responses (Lin et al., 2000, Yin et al., 2009). Both caspase-4 and -5 are functional inflammasome components. Caspase-4 mediates inflammasome activation in keratinocytes; caspase-5 does so in THP-1 cells; and both partially restrict L. pneumophila growth in THP-1 cells (Sollberger et al., 2012) (Martinon et al., 2002, Akhter et al., 2012). Our data indicate that caspase-4, not caspase-5, is required for IL-18 processing and secretion and pyroptotic cell death in human IECs. Caspase-4 is abundant in IECs (Figure 2.2), and only a minor increase in procaspase-4 is detected upon infection (Figure 2.4E). Although expression of caspase-11 is transcriptionally regulated by LPS in mouse macrophages and dendritic cells, it is constitutively expressed at high levels in the mouse intestine (Broz et al., 2012, Kayagaki et al., 2011) (Kang et al., 2004). The relative abundance of caspase-4 and -11, and their constitutive expression in epithelial cells and the intestine (Figure 2.2), might allow for the rapid sensing of enteric pathogens at mucosal sites (Demon et al., 2014, Kang et al., 2004).

The human epithelial noncanonical inflammasome is broadly responsive to intracellular and extracellular Gram-negative bacteria, a unique feature to date. LPS-containing outer membrane vesicles shed by extracellular enterohemorrhagic *E. coli* are internalized by colonic epithelial cells, potentially explaining how the inflammasome senses such pathogens (Bielaszewska *et al.*, 2013). In murine macrophages, caspase-1 is required for noncanonical inflammasome-mediated processing of IL-18 and IL-1β (Broz *et al.*, 2012, Kayagaki *et al.*,

2011). Our data indicate that caspase-11 affects IL-18 processing and secretion, but without testing $Casp1^{-/-}$ mice, we cannot rule out that caspase-1 is also required in murine IECs. By contrast, caspase-4-dependent IL-18 processing and secretion appears to be independent of caspase-1 in human IECs. Caspase-4 is able to cleave IL-18 at the same processing site as human caspase-1, which might account for our findings (Fassy *et al.*, 1998, Gu *et al.*, 1997). These distinctive features suggest there are mechanistic differences in noncanonical inflammasome activation between myeloid-derived cells and epithelial cells. Defining these features will be important for establishing the effector functions of caspase-4 and caspase-11 in different cell types and hosts.

Collectively, our work reveals a previously undiscovered host immune defense role for the inflammasome within IECs. Our results show that caspase-4 and -11 are triggered by enteric bacteria and drive inflammasome-based activation and release of IL-18, as well as pyroptotic epithelial cell death and shedding. Importantly, the actions of these inflammatory caspases limit pathogen colonization of the intestinal epithelium, representing a potent mechanism for antimicrobial host defense at mucosal surfaces.

2.4 Methods

2.4.1 Bacterial strains

Wild-type and $\Delta aroA$ Salmonella enterica serovar Typhimurium SL1344 (Hoiseth and Stocker, 1981, Mansson *et al.*, 2012), wildtype SL1344-harboring pFPV-mCherry, or pFPV25.1 for constitutive expression of mCherry or GFP, respectively (Drecktrah *et al.*, 2008, Valdivia and Falkow, 1996), and EPEC O127:H6 wildtype strain E2348/69 have been described previously

(Levine *et al.*, 1978). SL1344 *glmS::mCherry* and *glmS::gfpmut3* were created by site-specific insertion of *mCherry* (codon-optimized for *S*. Typhimurium) or *gfpmut3* at the *att*Tn7 site of the SL1344 chromosome using pGP-Tn7-Cm (Crepin *et al.*, 2012). Expression of the fluorescent proteins is under the control of the P*trc* promoter, which was amplified from pJC125 (Myeni *et al.*, 2013).

2.4.2 Cell culture

All cell lines were purchased from the American Type Culture Collection (ATCC). Caco-2 C2Bbe1 colorectal adenocarcinoma (ATCC CRL-2102), HCT-8 ileocecal colorectal adenocarcinoma (CCL-244), HCT 116 colorectal carcinoma (CCL-247), HeLa cervical adenocarcinoma (CCL-2), HT-29 colorectal adenocarcinoma (HTB-38), HuTu 80 duodenal adenocarcinoma (HTB-40), SW480 colorectal adenocarcinoma (CCL-228), and THP-1 monocytes (TIB-202) were grown as recommended by ATCC. THP-1 monocytes were differentiated with 200 nM phorbol myristic acid (PMA) for 24 hr. For polarization, C2Bbe1 cells were grown on collagen-coated cell culture inserts (1 μ m pore size, BD Falcon) in 24-well plates for 3 days as described (Knodler *et al.*, 2010). Monolayers with a transepithelial electrical resistance of \geq 250 Ω .cm² were used for infections.

2.4.3 siRNA knockdowns

C2Bbe1 cells (5×10^5 cells in 20 µl) were transfected in Nucleocuvette Strips (Lonza) with 2 µM siRNA using Nucleofector solution SE (Lonza) with an Amaxa 4D-Nucleofector (program CM-138) and then transferred to collagen-coated permeable cell culture inserts for 72 h prior to infection. HeLa cells were seeded in 6-well plates at 1.2×10^5 cells/well and transfected with

Dharma*FECT*1 reagent (Thermo Scientific) and 25 nM siRNA for 48 h prior to infection. ON-TARGETplus SMARTpool siRNA directed against human IL-18, caspase-1, -4, and -5 and a nontargeting pool were from Dharmacon (Thermo Scientific).

2.4.4 Nucleofection of plasmid DNA and LPS

Plasmids encoding human caspase-1, -4, and -5 were purchased from OriGene. Endotoxin-free plasmids were prepared with the Nucleobond Xtra Midi Plus EF Kit in accordance with the manufacturer's instructions (Macherey-Nagel). C2Bbe1 cells (4×10^5 cells in 20 µl Nucleofector solution SE) were nucleofected with 1 µg plasmid DNA as described above and then divided between two wells in a collagen-coated 24-well plate for 48 h prior to infection. Alternatively, cells were nucleofected with a dilution series of *S*. Typhimurium LPS (3–100 ng in cell-culture-grade water, Corning Cellgro) and then divided between two wells in a collagen-coated 24-well plate for 16 h prior to collection of cell-free supernatants.

2.4.5 Bacterial infections

Infection conditions for *S*. Typhimurium have been described previously (Knodler *et al.*, 2010). For EPEC infections, bacteria were grown static overnight at 37°C in 3 ml Luria-Bertani Miller (LB Miller) broth. An aliquot (100 μ l) of overnight culture was used to inoculate 5 ml Dulbecco's modified Eagle's medium (DMEM) and growth continued, statically, at 37°C in 10% CO₂for 3 hr. Cells were infected with 1 μ l EPEC subculture for 2.5 h at 37°C. Nonadherent bacteria were removed by washing eight times with Hank's balanced salt solution, and incubations continued in growth media containing 2 μ g/ml gentamicin.

2.4.6 Mouse strains and infections

 $Casp11^{-/-}$ and $Casp1^{-/-}$ $Casp11^{-/-}$ mice were obtained from Genentech (Kayagaki *et al.*, 2011). Asc^{-/-} and Nlrp3^{-/-} mice were obtained from Dr. Daniel Muruve (University of Calgary). $Nlrp3^{-/-}$ mice originated from the Department of Biochemistry and the Institute for Arthritis Research, University of Lausanne. C57BL/6 wildtype and knockout mice (8– 12 weeks old) were bred under specific pathogen-free conditions at the Child and Family Research Institute. For oral infections, mice were gavaged with streptomycin (100 mg/kg) 1 day before infection, then orally gavaged with an overnight LB culture containing $\sim 2.5 \times 10^6$ colonyforming units (CFU) of wildtype or $\Delta aroA S$. Typhimurium SL1344 (Strep^R, in some cases carrying pFPV25.1), and sacrificed at specified times p.i. (Mansson et al., 2012). For gallbladder infections, mice were intravenously injected with \sim 500 CFU of wildtype S. Typhimurium SL1344 (in some cases carrying pFPV25.1). For GFP-Salmonella infections, mice were given daily intraperitoneal injections of carbenicillin (100 mg/kg), beginning at 30min before the infection, to maintain the pFPV25.1 plasmid. All mouse experiments were performed in accordance with protocols approved by the University of British Columbia's Animal Care Committee and in direct accordance with the Canadian Council on Animal Care's guidelines.

2.4.7 Tissue collection, pathology scoring, and bacterial counts

Tissue collection and bacterial counts were as described previously (Knodler *et al.*, 2010, Mansson *et al.*, 2012). In brief, mice were euthanized, and the cecum, colon, or gallbladder were collected in 10% neutral-buffered formalin (Fisher) or 4% paraformaldehyde (Fisher) for histological analyses. For CFU counts, organs were collected separately; organs were homogenized in PBS (pH 7.4); and dilutions were plated on LB agar plates containing streptomycin. Cecal pathology was blindly scored by two researchers using hematoxylin and eosin-stained sections as previously described by Barthel *et al.* (2003) with the following modification: a score was also given for overall crypt loss within a cross-section (0, none; 1, up to 25% loss of crypts; 2, 26%–50% loss; 3, 51%–75% loss; 4, total loss). The cumulative scoring range for cecal inflammation (submucosal edema, PMN infiltration into the lamina propria, goblet cell loss, epithelial integrity, and overall crypt loss) was 0–17.

2.4.8 Statistical analysis

The mean \pm SD for at least three independent experiments is shown in all figures, unless stated otherwise. p values were calculated using a one-tailed Student's t-test or ANOVA with Dunnett's or Tukey's post-hoc test to compare means within the group. A p value of less than 0.05 was considered statistically significant.

2.5 Supplemental Experimental Procedures

2.5.1 Reagents

The following inhibitors were purchased from Enzo Life Sciences: Z-FA-FMK, Z-VADFMK, Z-D(OMe)E(OMe)VD(OMe)-FMK, Z-LE(OMe)HD(OMe)-FMK, Z-LE(OMe)VD(OMe)-FMK, Z-VD(OMe)VAD(OMe)-FMK, Z-VE(OMe)ID(OMe)-FMK, Z-WE(OMe)HD(OMe)-FMK, ZYVAD(OMe)-FMK. Stock solutions were prepared in DMSO and infected cells were treated with 20 µM inhibitor from 30min p.i. to 10h p.i. Rat tail collagen I was from BD Biosciences. Human transferrin, saponin, sodium deoxycholate and PMA were from Sigma-Aldrich. Quantitative Real-Time PCR (qPCR). RNA was extracted using TRIzol® (Life Technologies)

according to the manufacturer's instructions, with the modification that all centrifugation steps were carried out at 16,000 x g and the centrifugation at the precipitation step was increased to 30min. Phase lock tubes (5 PRIME) were used for the phase separation. RNA was resuspended in 20 µl nuclease-free water (Life Technologies). Alternatively, total RNA from human (21 week fetus) colonic epithelial cells and human (19 week fetus) small intestinal epithelial cells was purchased from ScienCell and DV Biologics, respectively. DNA was removed by DNase I treatment using the RapidOut DNA Removal Kit (Thermo Scientific) according to the manufacturer's instructions. RNA integrity was assessed by visualization of the 18S and 28S bands by agarose gel electrophoresis and SYBR® Safe DNA gel stain (Life Technologies). The purified RNA was quantified by using a Nanodrop 2000 spectrophotometer (Thermo Scientific). One µg of RNA was reverse transcribed to cDNA using Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific) according to the manufacturer's instructions. A no reverse transcriptase (NRT) control was performed for each RNA sample to verify the absence of DNA contamination. The cDNA was diluted 1:20 and an aliquot of 2 µl was used for each qPCR reaction. Amplifications were performed in duplicate by using Luminaris Color HiGreen qPCR Master Mix (Thermo Scientific) and fluorescence was read by a C1000 Touch Thermal Cycler, CFX96 Real-Time System (Bio Rad). A 20 µl reaction volume was used and the cycling protocol was 2min of UDG pre-treatment at 50°C, 10min of initial denaturation, followed by 40 cycles of 15s of denaturation at 95°C, 30s of annealing at 60°C or 62°C and 30s of extension at 72°C. Optimal PCR conditions and primer efficiencies (Table S1) were determined for each primer pair using a standard curve generated from a series of tenfold dilutions of a cDNA clone of the gene of interest (purchased from OriGene or Thermo Scientific). Specificity of the desired PCR product was determined by melting curve analysis. The following primer sequences were

selected from PrimerBank database (http://pga.mgh.harvard.edu/primerbank/): CASP1 (PrimerBank ID380254454c1), CASP4 (PrimerBank ID: 73622124c1) and CASP5 (PrimerBank ID: 209870072c1) (Wang and Seed, 2003). Ribosomal phosphoprotein P0 (RPLP0) was chosen as a reference gene based upon previous studies in intestinal epithelial cells, and the primer sequences were as described (Dydensborg *et al.*, 2006). IL-18 primers were designed using Primer-BLAST. For each gene, the Cq was normalized to that of the reference gene: Δ Cq = Cq(target gene) – Cq(reference gene). For siRNA mediated knockdown experiments, the Δ Cq was normalized to that of the non-targeting control sample: $\Delta\Delta$ Cq = Δ Cq(test sample) – Δ Cq(control sample). The expression or fold-change in expression of each gene was calculated as 2(- Δ Cq) or 2(- $\Delta\Delta$ Cq), respectively.

| Gene | Gene ID | Oligonucletide sequences 5'-3' | Amplicon size (bp) | Annealing temperature (°C) | Primer efficiency (%) | GenBank accession number of splice variants |
|-------|------------|----------------------------------|-----------------------|----------------------------------|-----------------------------|--|
| RPLP0 | 6175 | Forward: GCAATGTTGCCAGTGTCTG | 142 | 60 | 93 | NM_001002 |
| | | Reverse: GCCTTGACCTTTTCAGCAA | | | | NM_053275 |
| CASPI | 834 | Forward: TTTCCGCAAGGTTCGATTTTCA | 54 | 60 | 100 | NM_033292 |
| | | Reverse: GGCATCTGCGCTCTACCATC | | | | NM_001223 |
| | | | | | | NM_033293 |
| | | | | | | NM_033294 |
| | | | | | | NM_033295 |
| | | | | | | NM_001257118 |
| | | | | | | NM_001257119 |
| CASP4 | 837 | Forward: CAAGAGAAGCAACGTATGGCA | 267 | 60 | 93 | NM_001225 |
| | | Reverse: AGGCAGATGGTCAAACTCTGTA | | | | NM_033306 |
| CASP5 | 838 | Forward: TCACCTGCCTGCAAGGAATG | 92 | 62 | 92 | NM_001136109 |
| | | Reverse: TCTTTTCGTCAACCACAGTGTAG | | | | NM_001136110 |
| | | | | | | NM_001136112 |
| | | | | | | NM_004347 |
| IL-18 | 3606 | Forward: TGCCAACTCTGGCTGCTAAA | 104 | 60 | 90 | NM_001562 |
| | | Reverse: TTGTTGCGAGAGGAAGCGAT | | | | NM_001243211 |

Table 2.1 qPCR primers and conditions used in this study.

2.5.2 LPS Isolation

LPS was extracted from S. Typhimurium 14028 by the hot phenol/water method (Westphal, 1965). Freeze-dried bacteria were resuspended in endotoxin-free water at a concentration of 10 mg/ml. 12.5 ml of 90% phenol (Fisher Scientific) was added and the resultant mixture was vortexed and incubated in a hybridization oven at 65°C. The mixture was cooled on ice and centrifuged at 12, 096 x g at room temperature for 30minutes. The aqueous phase was collected, and an equal volume of endotoxin-free water was added to the organic phase. The sample was treated as above and aqueous phases were combined and dialyzed against Milli-Q purified water to remove residual phenol and then freeze-dried. The resultant pellet was resuspended at a concentration of 10 mg/ml in endotoxin-free water and treated with DNase at 100 µg/ml and RNase A at 25 µg/ml and incubated at 37°C for 1 hour in a water bath. Proteinase K was added to a final concentration of 100 µg/ml and incubated for 1 hour in a 37°C water bath (all enzymes sourced from Qiagen) (Fischer et al., 1983). The solution was then extracted with an equal volume of water-saturated phenol. The aqueous phase was collected and dialyzed against Milli-Q purified water and freeze-dried as above. The LPS was further purified by the addition of chloroform/methanol 2:1 [v:v] to remove membrane phospholipids and further purified by an additional water-saturated phenol extraction and 75% ethanol precipitation to remove contaminating lipoproteins (Folch et al., 1957, Hirschfeld et al., 2000). For structural analysis, 1 mg of purified LPS was converted to lipid A by mild-acid hydrolysis with 1% sodium dodecyl sulfate (SDS) (all other chemicals from Sigma, St Louis, MO) at pH 4.5 as described previously (Caroff et al., 1988).

2.5.3 MALDI-TOF mass spectrometry

Lipid A was analyzed on an AutoFlex Speed MALDI TOF mass spectrometer (Bruker Daltonics, Billerica, MA). Data was acquired in reflectron negative and positive modes with a Smartbeam laser with 1 kHz repetition rate and up to 500 shots were accumulated for each spectrum. Instrument calibration and all other tuning parameters were optimized using Agilent Tuning mix (Agilent Technologies, Foster City, CA). Data was acquired and processed using flexControl version 4.0 and flexAnalysis version 3.3 (Bruker Daltonics, Billerica, MA).

2.5.4 Enumeration of intracellular bacteria

Infected monolayers were washed once in phosphate buffered saline (PBS), then solubilized in 0.5-1 ml 0.2% sodium deoxycholate in PBS. Serial dilutions were plated on LB agar for enumeration of intracellular *S. Typhimurium*. Caspase activity and SYTOX® Orange staining. Mock-infected or infected epithelial cells were incubated with FAM-YVAD-FMK FLICATM reagent in cell culture medium for 1h at 37°C in 10% CO2 according to the manufacturer's instructions (Immunochemistry Technologies). Cells were washed once in PBS, fixed in 3.5% PFA and DNA stained with 1 μ g/ml Hoechst 33342 (Life Technologies) and 1 μ g/ml Hoechst 33342 (Life Technologies)

2.5.5 Fluorescence microscopy (*in vitro*)

Monolayers infected with *S*. Typhimurium pFPV-mCherry were fixed, permeabilized and immunostained for the tight junction protein, ZO-1 (rabbit anti-ZO1 (mid) polyclonal antibody, Life Technologies; Alexa Fluor 488 goat anti-rabbit IgG, Life Technologies). DNA was stained with Hoechst 33342 (1 μ g/ml; Life Technologies), then cell culture inserts were excised and mounted on a glass slide in Prolong Gold Antifade (Life Technologies). Samples were viewed on a Leica DM4000 upright fluorescent microscope. For quantification of extrusion, an extruding cell was scored as positive if its nucleus was above the plane of the monolayer and/or had a ZO-1 rosette at its base (Corfe *et al.*, 2000, Knodler *et al.*, 2010, Rosenblatt *et al.*, 2001).

2.5.6 Fluorescence microscopy (*in vivo*)

Immunofluorescence staining of uninfected and infected tissues was performed using previously described procedures (Knodler *et al.*, 2010, Mansson *et al.*, 2012). In brief, cecal tissues were fixed in 4% paraformaldehyde for 2 h, rinsed in 0.1% sodium azide/PBS and incubated overnight in 20% sucrose/PBS. Tissues were then embedded in Cryomatrix (Thermo Scientific), frozen with isopentane (Sigma-Aldrich) in a dry ice bath and stored at -20° C. Serial sections were cut (6 µm) and washed with PBS azide. Gallbladders were fixed in 10% neutral buffered formalin (Fisher) for 16 h, rinsed in 70% ethanol and paraffin embedded, (sectioning was completed by the histology laboratory at the BC Children's Hospital Research Institute). Serial sections were cut (5 µm) and deparaffinized by heating at 60°C, xylene treatment and rehydration through an ethanol gradient to water. Sections were treated with PBS containing 0.1% Triton X-100, followed by blocking buffer (PBS containing 0.05% Tween 20 and 1% bovine serum albumin (BSA) for GFP detection, or 1% normal donkey serum for cytokeratin immunostaining).

Endogenous biotin was blocked as described by the manufacturer (Life Technologies). For GFP detection, biotinylated anti-GFP antibodies (1:300, Genetex) were used followed by Alexa Fluor 488-conjugated streptavidin (1:1000, Life Technologies) and Alexa Fluor 568-conjugated phalloidin (1:500, Life Technologies). For immunodetection of epithelial cells, tissues were incubated with a goat anti-cytokeratin 19 polyclonal antibody (1:300, Santa Cruz Biotechnology) or rat anti-CD326 (EpCAM) (1:300, eBioscience) followed by incubation in biotin blocking buffer. GFP immunostaining then proceeded as described above. Tissues were then probed with Alexa Fluor 488-conjugated streptavidin and Alexa Fluor 568-conjugated donkey anti-goat IgG or Alexa Fluor 568-conjugate goat anti-rat IgG (1:1000; Life Technologies). Tissues were mounted using ProLong Gold Antifade reagent (Life Technologies) containing DAPI for DNA staining. Sections were viewed on a Zeiss AxioImager microscope and images taken using an AxioCam HRm camera operating through AxioVision software.

2.5.7 Immunoblotting (in vitro)

For analysis of whole cell lysates, adherent cells were washed once in PBS prior to lysis in boiling 1.5x SDS-PAGE sample buffer. For detection of IL-18 released into the culture supernatant, infected cells were incubated in serum-free media containing 10 µg/ml gentamicin from 90min p.i. Supernatants were collected at 10h p.i. and proteins precipitated overnight at 4°C with 10% (v/v) trichloroacetic acid. Samples were centrifuged at 16,000 x g for 20min at 4°C. Pellets were washed in cold acetone and resuspended in boiling 1.5x SDS-PAGE sample buffer. Proteins were separated by SDS-PAGE, transferred to nitrocellulose (BioRad) and immunoblotting was as described previously with the following antibodies: rabbit polyclonal anti-caspase-1 (A-19) (1:2,000; Santa Cruz Biotechnology), mouse monoclonal anti-caspase-4 (4B9) (1:2,000; Santa Cruz Biotechnology), rabbit polyclonal anti-human IL-18 (1:10,000;
MBL), rabbit polyclonal anti-human IL-1β (1:2,000; Abcam), mouse monoclonal anti-β-actin
(8H10D10) (1:20,000; Cell Signalling Technology) (Knodler *et al.*, 2009).

2.5.8 Immunoblotting (in vivo)

For analysis of cecal tissue lysates, tissue was collected, washed twice in PBS then divided into three. One third was frozen at -80°C and the other two were incubated in Cell Recovery Solution (BD) for 2h at 4°C. After incubation, crypts were dislodged by tissue agitation and separated from the lamina propria fraction by aspiration. Both fractions were centrifuged at 350 x g for 5min, supernatant was discarded and samples were stored at - 80 °C. Crypt secretions were prepared by resuspending fresh crypt fraction in 150 µl of DMEM (Invitrogen) (plus penicillinstreptomycin and gentamicin), incubating for 3h at 37°C, 5% CO2, centrifuged at 16,000 x g for 20min at 4°C, the supernatant collected and stored at -80°C. To prepare lysates, tissues were homogenized in RIPA buffer with cOmplete protease inhibitors (Roche) and incubated on ice, with agitation, for 2 h. Samples were then centrifuged at 16,000 x g for 20min at 4°C, normalized to 30 µg total protein, 2x SDS-PAGE sample buffer added and boiled for 5min. Proteins were separated by 15% SDS-PAGE, transferred to Hybond-P PVDF membrane (GE), followed by immunoblotting with the following antibodies: rabbit polyclonal anti-caspase-1 (M-20) (1:1,000; Santa Cruz Biotechnology), rat monoclonal anti-caspase-11 (17D9) (1:100; Santa Cruz Biotechnology), rabbit polyclonal anti-mouse IL-18 (1:1,000; Rockland), rabbit polyclonal anti-mouse IL-1 β (1:1,000; Genetex), rabbit monoclonal anti- β -actin (13E5) (1:5,000; Cell Signalling Technology) and goat anti-cytokeratin-19 (M-17) (1:1,000; Santa Cruz Biotechnology).

2.5.9 Cytokine measurements

Culture supernatants were collected (when collected from polarized cells grown on transwells: from either the top (apical) or bottom (basolateral) receptacle) and centrifuged at 12,000 x g for 10min at 4°C. IL-8 and IL-18 were quantified using commercially available ELISA kits for human IL-8 (R&D Systems) and human IL-18 (MBL). According to the manufacturer, the MBL ELISA has less than 1% cross-reactivity with pro-IL-18, which has been independently confirmed (Melnikov *et al.*, 2001). Alternatively, IL-18 was measured using a sandwich ELISA comprising mouse monoclonal antibody 125-2H (MBL) as the capture antibody and biotinlabeled 159-12B rat monoclonal (MBL) as the detection antibody. These antibodies react exclusively with mature IL18 (Akita *et al.*, 1997, Taniguchi *et al.*, 1997). An IL-18 standard curve of 7.8-500 pg/ml was generated by serial dilution of recombinant human IL-18 (MBL). Cecal tissues were removed from mice 3 days after infection with *S*. Typhimurium. Tissues were washed free of luminal contents and then incubated in DMEM (supplemented with penicillinstreptomycin and gentamicin) for 6 h. Supernatants were collected and IL-18 and IL-1β levels were measured by ELISA (MBL and R&D Systems) following the manufacturer's protocols. Chapter 3: Intestinal restriction of *Salmonella enterica* serovar Typhimurium requires caspase-1 and caspase-11 epithelial intrinsic inflammasomes

3.1 Introduction

Within the mammalian gastrointestinal (GI) tract, intestinal epithelial cells (IECs) provide the primary interface between the microbial-rich gut lumen and the underlying mucosal immune system. Here they play a central role in the coordination of mucosal homeostasis, tempering proinflammatory responses while remaining rapidly reactive to noxious stimuli such as enteric pathogens. One recently described mechanism by which IECs engage in immune defense is through the activation of cell-intrinsic inflammasomes that require inflammatory caspases, namely caspase-1 and caspase-11 in mice, or caspase-1 and caspase-4 in humans (Knodler *et al.*, 2014, Sellin *et al.*, 2014).

During the initial stages of an enteric infection, *Salmonella enterica* serovar Typhimurium (*S*. Typhimurium) migrates from the gut lumen towards the intestinal epithelium, subsequently invading IECs. The invasion and intracellular proliferation of this pathogen triggers the activation of IEC-intrinsic inflammasomes, resulting in the expulsion of infected IECs into the intestinal lumen. The more rapidly these cells are shed, the less time is available for intracellular *Salmonella* to proliferate and invade surrounding IECs or translocate into the underlying lamina propria. In 2014, Sellin and colleagues showed this process requires the Nod-like receptors (NLRs) Naip1-6 and Nlrc4 (Sellin *et al.*, 2014), which form an inflammasome platform that activates caspase-1. During the early stages of a *S*. Typhimurium infection (12 h post-infection
(p.i.)), the IECs lining the ceca of $Naip1-6^{-/-}$ and $Nlrc4^{-/-}$ mice were found to be heavily infected, containing densely packed microcolonies of intracellular *Salmonella* (up to 20 bacteria per cell), which were only rarely observed in the IECs of wildtype mice (Sellin *et al.*, 2014). Through bone marrow transplantation studies, as well as the use of $Naip1-6^{AIEC-/-}$ mice, the authors demonstrated this microcolony phenotype was caused by the loss of Naip-Nlrc4 inflammasome activation in IECs. Notably, the protective role for Naip1-6 was acute as the $Naip1-6^{AIEC-/-}$ mice and wildtype mice showed no difference in *Salmonella* colonization of the cecum or histopathology at later time points (36 h p.i.)

In the Sellin *et al.* study, *Salmonella* loads in the mucosa of $Casp1/11^{-/-}$ mice were intermediate between that of *Naip1-6^{-/-}* or *Nlrc4^{-/-}* mice and wildtype mice, whereas $Casp11^{-/-}$ mice phenocopied wildtype mice at 18 h p.i. (Sellin *et al.*, 2014). In an independent study, we demonstrated that a non-canonical inflammasome involving caspase-11 is was involved at later time points during enteric *S*. Typhimurium infection in mice (Knodler *et al.*, 2014). Specifically, $Casp11^{-/-}$ mice carried higher *Salmonella* loads in the cecum and cecal lumen at 7 days p.i. and displayed an intracellular IEC microcolony phenotype similar to that described by Sellin *et al.* at 24 h p.i. (Knodler *et al.*, 2014). Importantly, this phenotype was also observed in the gallbladder of $Casp11^{-/-}$ mice, indicating that caspase-11 dependent control of epithelial cell shedding is not restricted to the large intestine.

Thus the reports by Sellin *et al.* and Knodler *et al.* both detailed an important connection between epithelial-intrinsic inflammasome activation, cell shedding and intracellular bacterial burdens (Sellin *et al.*, 2014, Knodler *et al.*, 2014). However, the individual contributions and potential functional overlap of caspase-1 and caspase-11 to host protection against *Salmonella* in the gut has yet to be determined, primarily because mice deficient only in caspase-1 were not available. Recently this has changed, as $Casp1^{-/-}$ mice have been generated by a handful of groups (Rauch *et al.*, 2017, Man *et al.*, 2017, Schneider *et al.*, 2017). To define the exact involvement of caspase-1 and caspase-11 in antimicrobial defenses within the gut, we directly compared *S*. Typhimurium colonization in $Casp1^{-/-}$, $Casp11^{-/-}$ and $Casp1/11^{-/-}$ mice as well as in enteroids. Our results demonstrate that caspase-1 primarily regulates inflammasome responses in IECs at baseline whereas caspase-11 plays a compensatory role upon extrinsic stimulation of inflammatory signaling pathways in IECs. Therefore, canonical and non-canonical IEC-intrinsic inflammasomes cooperate to provide an important innate immune defense against pathogen infections.

3.2 Results

3.2.1 Inflammasome-deficient mice carry higher intestinal tissue and luminal *Salmonella* burdens

To define the exact contributions of caspase-1 and caspase-11 to enteric host defense, we infected C57BL/6 (wildtype), $Casp1^{-/-}$, $Casp11^{-/-}$ and double-deficient $Casp1/11^{-/-}$ mice with *S*. Typhimurium via the orogastric route. The $Casp1^{-/-}$ and $Casp1/11^{-/-}$ mice proved highly susceptible to infection, carrying heavy cecal, colonic and luminal pathogen burdens at 18 h p.i. (Figure 3.1A). Although their cecal tissue burdens were not as high as those carried by the $Casp1^{-/-}$ and $Casp1/11^{-/-}$ mice, the $Casp11^{-/-}$ mice also displayed significantly higher intestinal and luminal burdens than wildtype mice at 18 h p.i. (*, P < 0.05, Figure 3.1A) and their intestinal burdens remained high at 72 h p.i. (Figure 3.1A). Interestingly, wildtype cecal burdens displayed

a marked seven-fold decrease between 18 h and 72 h p.i. whereas only a minor decrease was observed in the $Casp1^{-/-}$ and $Casp1/11^{-/-}$ mice, while $Casp11^{-/-}$ intestinal burdens remained comparable to those at 18 h p.i. These changes were also reflected in the 72h p.i. colonic burdens with the $Casp11^{-/-}$ levels remaining high, however the decreases observed in the $Casp1^{-/-}$ and $Casp1/11^{-/-}$ levels remaining high, however the decreases observed in the $Casp1^{-/-}$ and $Casp1/11^{-/-}$ mice were much more dramatic, albeit significantly higher than wildtype (Figure 3.1A). This suggests the inflammatory caspase-deficient mice were unable to clear the infection from their tissues as efficiently as wildtype mice, a finding corroborated by their higher fecal shedding burdens (Figure 3.2). Expression profiles of *Casp1* and *Casp11* in the cecal tissues of wildtype mice revealed that *Casp11* transcripts increased over the course of infection, while *Casp1* levels decreased (Figure 3.1B), which is consistent with other reports (Sellin *et al.*, 2014, Schauvliege *et al.*, 2002, Eva *et al.*, 2014).



Figure 3.1 Inflammatory caspases are required for the epithelial restriction of a *Salmonella* infection *in vivo*. Streptomycin-pretreated C57BL/6 (wildtype), *Casp11^{-/-}*, *Casp1^{-/-}* and *Casp11^{-/-}*(*Casp11^{-/-}*(*Casp11/11^{-/-}*) mice were orally infected with *S*. Typhimurium $(3 \times 10^6 \text{ CFU})$, with intestinal tissue and luminal contents plated at 18 h post infection (p.i.) and 72 h p.i. (A). *Casp11* and *Casp1* gene expression enumerated relative to *Rplp0* reference from cecal RNA of streptomycin pretreated controls, 18h p.i. and 72h p.i., wildtype and *Casp1/11^{-/-}* mice. (B). Representative fluorescence images of infected cecal tissues at 18 h p.i. *Salmonella* (red), E-cadherin (green), and DNA (blue) (C). Original magnification ×200, Inset ×630; scale bars 50µm, inset scale bars 5µm. Asterisk denotes presence of intracellular *Salmonella* (L.u. denotes cecal lumen). The number of *Salmonella*-infected IECs per crypt (D), the number of intracellular *Salmonella* in each infected IEC (E) and the proportion of apically shedding IECs adjacent to infected crypts (F). Statistical significance for 1A calculated using Mann-Whitney U-test (*p<0.05; **p<0.01; ***p<0.001) with student *t*-test (*p<0.05; ***p<0.001) applied to 1B and 1D-F. Each symbol represents one animal. Mean and SEM are indicated. Results are from at least two independent experiments. Blinded 18 h p.i. cecal tissues analyzed in 1D-F were from n = 5 mice (with 50 representative crypts scored) from at least two independent experiments.



Figure 3.2 Enumeration of luminal *Salmonella* **shed in fecal pellets at 24h and 48h p.i.** Streptomycin-pretreated wildtype, $Casp11^{-/-}$, $Casp1^{-/-}$ and $Casp1/11^{-/-}$ mice were orally infected with *S*. Typhimurium (3 × 10⁶ CFU) and stool collected at 24 h and 48 h p.i. and plated to enumerate *Salmonella* shedding. Each symbol represents one animal. Mean and SEM are indicated. Results are from at least two independent experiments. Statistical significance was calculated using Mann-Whitney U-test *p<0.05; **p<0.01; ***p<0.001.

3.2.2 Inflammasome-deficient mice display increased numbers of infected IECs and

higher intracellular Salmonella burdens

To investigate if the increased intestinal burdens recovered from the caspase-deficient mice indicated potential differences in tissue localization, we immunofluorescently stained infected cecal tissues (18 h p.i.) for *S*. Typhimurium. In all mouse backgrounds, the majority of the *Salmonella* were confined to the cecal lumen, however a small intraepithelial subset was also

observed (Figure 3.1C). Focusing on this intracellular subset, we noted that the cecal crypts of wildtype mice remained relatively sterile, with only an occasional infected cell identified per crypt (fewer than 10% of crypts carried infected IECs at 18 h p.i.) (Figure 3.1D). Of the infected IECs, they contained only 1-2 *Salmonella* per cell on average (Figure 3.1E). Moreover, these infected wildtype IECs were largely confined to the tips of crypts or in the process of being actively shed from the epithelial surface (Figure 3.1C), similar to that described previously (Sellin *et al.*, 2014, Knodler *et al.*, 2014). In contrast, all the inflammatory caspase-deficient mice showed increased numbers of *Salmonella*-infected IECs (Figure 3.1C; 22%, 38% and 42% of the cecal crypts of *Casp11^{-/-}*, *Casp1^{-/-}* and *Casp1/11^{-/-}* mice, respectively, showed infected IECs). *Casp1^{-/-}* and *Casp1/11^{-/-}* mice exhibited both the highest number of infected IECs/crypt (Figure 3.1D) (mean of 2-3 infected IECs/crypt), but also the highest number of *Salmonella* per IEC, with numerous IECs containing microcolonies comprising ≥ 10 bacteria (Figure 3.1E).

3.2.3 Wildtype mice experience high levels of IEC shedding largely localized to infected crypts

To address the connection between IEC shedding and *S*. Typhimurium invasion *in vivo*, 18 h p.i. cecal tissues were scored for epithelial damage. Whereas the ceca of wildtype mice demonstrated widespread signs of crypt and IEC deterioration, the cecal epithelium of $Casp1^{-/-}$, $Casp11^{-/-}$ and $Casp1/11^{-/-}$ mice was largely intact, as were their cecal crypts (Figure 3.3). While small numbers of shed IECs were found in the ceca of all the infected inflammatory caspase-deficient mice, the degree of IEC shedding was exaggerated in infected wildtype mice (Figure 3.1F; Figure 3.3). For example, wildtype mice displayed severe erosion of their epithelial surface with increased IEC shedding at most apical tips (Figure 3.3). In contrast, the cecal epithelium of

 $Casp1^{-/-}$ and $Casp1/11^{-/-}$ mice demonstrated only minor desquamation whereas $Casp11^{-/-}$ mice presented an intermediate phenotype, where the majority of crypts displayed only minor damage but with a modest increase in IEC shedding (Figure 3.3). Of note, in wildtype mice there was a strong correlation between the presence of an infected crypt and local IEC shedding, while this relationship was minimal in the ceca of the caspase-deficient mice (Figure 3.1F).



Figure 3.3 IEC histopathological damage from acute 18h p.i. Salmonella infection.

Streptomycin-pretreated wildtype, $Casp1^{-/-}$, $Casp1^{-/-}$ and $Casp1/11^{-/-}$ mice were orally infected with *S*. Typhimurium (3 × 10⁶ CFU) and epithelial integrity in cecal tissues at 18 h p.i. scored blinded. (**A**). Representative H&E staining of cecal tissue from streptomycin-pretreated wildtype, $Casp11^{-/-}$, $Casp1^{-/-}$ and $Casp1/11^{-/-}$ mice at 18 h p.i. n = 5 per a genotype. (**B**). Arrows denote IECs that are actively shedding or have been shed. Original magnification ×200; scale bars 100 µm. Statistical significance was calculated using student *t*-test *p<0.05; ***p<0.001.

3.2.4 *Casp1^{-/-}* and *Casp1/11^{-/-}* enteroid-derived monolayers exhibit increased numbers of

infected IECs and higher intracellular Salmonella burdens

To define whether the ability of inflammatory caspases to restrict S. Typhimurium infection in

murine ceca reflected an IEC intrinsic role, or alternatively, confounding factors such as the

intestinal microbiota or infiltrating immune cells that may alter IEC function, we generated cecal enteroids from uninfected mice. After generating 2D monolayers from these enteroids, they were infected with S. Typhimurium and intracellular bacteria were enumerated using a gentamicin protection assay. $Casp 1^{-/-}$ and $Casp 1/11^{-/-}$ monolayers proved highly susceptible to Salmonella infection, with immunostaining revealing large numbers of infected IECs which, despite being infected, remained intact within the monolayer (Figure 3.4A and 2B). Moreover, many of these infected cells contained large microcolonies of intracellular Salmonella, with some IECs containing over 100 bacteria (Figure 3.4C). In contrast, wildtype and $Casp11^{-/-}$ monolayers (Figure 3.4A-C), showed stronger responses to infection, with significantly fewer infected adherent IECs (Figure 3.4B) as well as relatively low numbers of intracellular Salmonella (Figure 3.4C). This result is consistent with a previous report that caspase-11 is not required for S. Typhimurium restriction within IECs in vivo (Sellin et al., 2014). With the exception of the *Casp11^{-/-}* monolayers, intracellular burdens in enteroids largely mirrored the *in vivo* findings that inflammatory caspase-deficient mice carried higher intracellular Salmonella levels than wildtype mice (Figure 3.4C).



Figure 3.4 Epithelial intrinsic inflammasomes restrict the intracellular proliferation of *Salmonella* predominantly through caspase-1 induced cell shedding and death.

Cecal enteroid monolayers derived from wildtype, $Casp11^{-/-}$, $Casp1^{-/-}$ and $Casp1/11^{-/-}$ mice were infected with *S*. Typhimurium (1:50 MOI) for 10 h. Representative fluorescence images depicting *Salmonella* (red), actin (green) and DNA (blue) (**A**). Original magnification ×400; scale bars 50µm. Arrows denote actively shedding or shed IECs and asterisks denote large foci of intracellular *Salmonella*. The severity of infection was also determined by the percentage of adherent infected IECs (**B**) and the number of intracellular *Salmonella* in each infected IEC (**C**). Results are from at least 400 IECs from two independent experiments. Representative fluorescence images utilized in cell shedding enumerations *Salmonella* (red), actin (green) and DNA (blue) (**D**). Original magnification ×200; scale bars 50µm. Percentage of shed/shedding IECs from the monolayer (from at least four blinded fields of view from two independent experiments) (**E**). IEC cytotoxicity as measured by lactate dehydrogenase activity released into the growth media at 10 h p.i. (**F**). Mean and SEM indicated from duplicate wells in two independent experiments. Statistical significance calculated with student *t*-test n.s. p>0.05; *p<0.05; **p<0.01; ***p<0.01.

3.2.5 Wildtype and *Casp11^{-/-}* enteroid-derived monolayers display increased cell

shedding and death

We have previously observed the shedding of *Salmonella*-infected IECs *in vivo* and *in vitro* (Knodler *et al.*, 2010, Knodler *et al.*, 2014). Shedding IECs were also evident in our murine enteroid infection model, in agreement with a recent study of *S*. Typhimurium infection of human enteroids (Co *et al.*, 2019). Compared to adherent IECs, shedding IECs presented a markedly different cell morphology; characterized by small, condensed DNA (leading to a comparatively stronger DAPI/nuclei signal), a 'ruffled' cytoskeletal actin signal and a slightly higher z-axis location in the monolayer, whereas adherent IECs displayed larger nuclei and clear cell-to-cell junction 'lattice-like' actin staining (Figure 3.4A and D) (Knodler *et al.*, 2010). To quantify cell shedding, the size and intensity of DAPI signals were evaluated. IEC shedding was found to be significantly enhanced upon infection in wildtype and *Casp11^{-/-}* monolayers as compared to modest shedding in *Casp1^{-/-}* and *Casp1/11^{-/-}* cells (Figure 3.4D and E). The proportion of infected shed IECs compared to infected adherent IECs was also comparatively higher in wildtype and *Casp11^{-/-}* monolayers (Figure 3.5). Similarly, wildtype and *Casp11^{-/-}* monolayers exhibited higher levels of cytotoxicity as measured by the release of the cytosolic

enzyme, lactate dehydrogenase, into the growth media (Figure 3.4F). These results support the concept that inflammatory caspases promote the expulsion of infected, dying IECs into the gut lumen (Knodler *et al.*, 2010, Sellin *et al.*, 2014, Knodler *et al.*, 2014).



Figure 3.5 Enumeration of shed IECs 10h after Salmonella infection.

Cecal enteroid monolayers derived from wildtype, $Casp11^{-/-}$, $Casp1^{-/-}$ and $Casp1/11^{-/-}$ mice were infected with *S*. Typhimurium (1:50 MOI) for 10 h and the percentage of shed infected IECs enumerated. Results are from at least 400 IECs from two independent experiments. Statistical significance was calculated using student *t*-test n.s. p>0.05; **p<0.01; ***p<0.001.

3.2.6 Inflammasome activation is only detected in *Salmonella*-infected shedding cells and

is absent in *Casp1/11^{-/-}* monolayers

We previously showed that Salmonella-infected human IECs undergoing extrusion label for

active caspase-1 and -4 (Knodler et al., 2010). To detect inflammatory caspase activity in

infected murine enteroids we employed a cell permeable fluorescent caspase activity dye (660-

YVAD-FMK FLICA), which covalently couples to active caspase-1 and caspase-11. By

fluorescence microscopy, wildtype monolayers exhibited strong FLICA signals for active

caspase-1/11 in infected cells undergoing shedding (Figure 3.6). This phenotype was also seen in

 $Casp1^{-/-}$ and $Casp11^{-/-}$ monolayers, despite overall lower numbers of shedding IECs in the $Casp1^{-/-}$ monolayers. In contrast, active caspase-1/11 was not detected in the $Casp1/11^{-/-}$ monolayers (Figure 3.7). The FLICA signal in wildtype, $Casp1^{-/-}$ and $Casp11^{-/-}$ monolayers appeared diffuse throughout the cell cytoplasm, while small high-intensity puncta were also observed, but only in wildtype and $Casp11^{-/-}$ monolayers (Figure 3.6), appearing similar to FLICA-positive signals described for *Nlrc4*-canonical inflammasome formation in macrophages (Man *et al.*, 2013). These results suggest that the antimicrobial activity of caspase-1 is dominant in IECs, but a caspase-11 inflammasome is also present, although more evident in cells lacking caspase-1 function.



Figure 3.6 Shedding IECs demonstrate high levels of inflammatory caspase activity.

Cecal enteroid monolayers generated from wildtype, $Casp11^{-/-}$, $Casp1^{-/-}$ and $Casp1/11^{-/-}$ mice were infected with *Salmonella* (1:50 MOI) for 10 h. (A) Representative fluorescence image depicting inflammatory caspase activity (FAM-YVAD-FMK activity; green), *Salmonella* (red), actin (white) and DNA (blue). Original magnification ×400; scale bars 25µm.



Figure 3.7 Over-exposed fluorescence image of *Casp1/11^{-/-}* monolayer.

Cecal enteroid monolayers were infected with *S*. Typhimurium (1:50 MOI) for 10 h. Over-exposed fluorescence image of $Casp1/11^{-/-}$ monolayer depicting an overall lack of inflammatory caspase activity (FAM-YVAD-FMK activity; green; 10X exposure time compared to Figure 3.6) in shedding IECs heavily infected with *Salmonella* (red), actin (white) and DNA (blue). Original magnification ×400; scale bars 25µm.

3.2.7 IFN-γ priming functionally differentiates enteroid monolayers derived from

Casp1^{-/-} and Casp1/11^{-/-} mice in terms of Salmonella-induced cell shedding

We hypothesized that the limited role for caspase-11 in enteroid-derived monolayers could be due to a lack of extrinsic factors present during *in vivo* infection of mice, such as inflammatory cytokines and chemokines that directly modulate IEC expression of innate defense proteins (Allaire *et al.*, 2018). Interferon (IFN)- γ is a potent cytokine released by immune cells which induces hundreds of genes promoting host defense (MacMicking, 2012, Eva *et al.*, 2014). It helps drive mucosal inflammation during the late stages of *Salmonella* colitis and has been described as an early stage effector cytokine with high systemically circulating levels during the first day of oral infection (Spees *et al.*, 2014, Dolowschiak *et al.*, 2016). When we analyzed cecal tissues collected from streptomycin-pretreated uninfected and *S*. Typhimurium-infected mice at 18 h and 72 h p.i. we noted that IFN- γ protein levels were significantly elevated in all infected genotypes at 18 h p.i., as compared to uninfected control tissues (Figure 3.8). Interestingly, IFN- γ levels remained high in the infected ceca of wildtype mice at 72 h p.i., significantly lower levels were produced by the inflammatory caspase-deficient mice, particularly the *Casp1*^{-/-} and *Casp1/11^{-/-}* mice. To test whether IFN- γ could alter inflammatory caspase production in IECs, pro-caspase-1 and pro-caspase-11 levels in naïve and IFN- γ treated wildtype cecal enteroids were compared by immunoblotting. High levels of caspase-1 were present irrespective of IFN- γ treatment, whereas caspase-11 levels increased upon IFN- γ treatment (Figure 3.9A). This suggests that activity of the non-canonical inflammasome might be potentiated in murine IECs as part of the host inflammatory response to infection.



Figure 3.8 *Ex vivo* cecal secretion of IFN- γ 18h and 72h p.i. with *Salmonella*.

Streptomycin-pretreated wildtype, $Casp11^{-/-}$, $Casp1^{-/-}$ and $Casp1/11^{-/-}$ mice were orally infected with *S*. Typhimurium (3 × 10⁶ CFU) for 18 h and 72 h p.i., ceca collected and transferred to secretion media for 24 h. Streptomycin-pretreated wildtype and $Casp1/11^{-/-}$ uninfected ceca were also collected as controls (Ctrl). *Ex vivo* secretions were measured by ELISA for murine IFN- γ . Each symbol represents one animal. Mean and SEM are indicated. Results are from at least two independent experiments. Statistical significance was calculated using student *t*-test *p<0.05; ***p<0.001.



Figure 3.9 IFN-γ pretreated enteroid monolayers display enhanced levels of IEC shedding and a dependence on caspase-1 and caspase-11 for restriction of *Salmonella* infection.

Enteroids generated from wildtype, $Casp11^{-/-}$, $Casp1^{-/-}$ and $Casp1/11^{-/-}$ mice were treated for 16 h with vehicle control or IFN- γ (10 ng/mL), then cell lysates probed for pro-caspase-1 (p46), pro-caspase-11 (p43) or actin by Western blotting. Densities relative to actin are shown (**A**). Cecal enteroid monolayers from wildtype, $Casp11^{-/-}$, $Casp1^{-/-}$ and $Casp1/11^{-/-}$ mice were either pretreated with IFN- γ (10 ng/mL) or vehicle control 16 h prior to infection with *S*. Typhimurium (1:50 MOI) for 10 h. Representative fluorescence images depicting *Salmonella* (red), actin (green) and DNA (blue) of cecal enteroid monolayers 10 h p.i. either with IFN- γ or vehicle control pretreatment (**B**). Original magnification ×400; scale bars 50µm. Arrows denote actively shedding or shed IECs and asterisks denote large foci of intracellular *Salmonella*. Percentage of shed/shedding IECs (from five blinded fields of view from two independent experiments) (**C**). The number of intracellular *Salmonella* in each infected IEC either primed with IFN- γ or vehicle control (**D**). Results are from 400 IECs from two independent experiments. Statistical significance calculated with student *t*-test ***p<0.001.

Next, we assessed the effect of IFN- γ -priming on the enteroid monolayer response to infection. IFN- γ pretreatment alone had no overt effect on cell shedding under basal conditions in all genotypes (Figure 3.10). However, IFN- γ pretreatment followed by *S*. Typhimurium infection significantly increased cell shedding in wildtype and $Casp1^{-/-}$ enteroid monolayers (Figure 3.9B and C). In contrast, $Casp11^{-/-}$ and $Casp1/11^{-/-}$ monolayers were not affected by IFN- γ priming, with $Casp11^{-/-}$ monolayers maintaining high levels of cell shedding that were not significantly different from their infected control, while $Casp1/11^{-/-}$ monolayers exhibited little IEC shedding even after pretreatment with IFN- γ (< 2% of total IECs) (Figure 3.9B and C).



Figure 3.10 IFN- γ **pretreatment alone had no overt effect on cell shedding for baseline conditions** Cecal enteroid monolayers from wildtype, $Casp11^{-/-}$, $Casp1^{-/-}$ and $Casp1/11^{-/-}$ mice were generated and either treated with media control (DMEM) or IFN- γ (10ng/mL) for 16h, then media changed to infection control conditions for 10h. Percentage of shed/shedding IECs (from five blinded fields of view from two independent experiments) was enumerated. Statistical significance was calculated using one-way ANOVA; no significant difference was determined between samples.

We then assessed if increased IEC expression of caspase-11 upon IFN- γ pretreatment impacted *S*. Typhimurium infection. Mirroring the cell shedding phenotype, IFN- γ priming decreased the mean number of intracellular *Salmonella* per cell in wildtype and *Casp1^{-/-}* enteroid monolayers as compared to no IFN- γ treatment (Figure 3.9B and D). Strikingly, IFN- γ pretreatment of *Casp1^{-/-}* monolayers nearly eliminated intracellular *Salmonella* microcolony formation (\geq 100 bacteria/cell) to less than 1% of all infected IECs (Figure 3.9D). By contrast, the mean number of intracellular *Salmonella* per cell was unchanged for *Casp11^{-/-}* and *Casp1/11^{-/-}* monolayers after IFN- γ pretreatment (Figure 3.9B and D). Overall, these findings indicate that extrinsic stimuli, such as IFN- γ , promote caspase-11, but not caspase-1 function in IECs.

3.3 Discussion

Through the use of *in vivo* mouse infections as well as enteroid-derived monolayers, we have deciphered the molecular wiring regulating the IEC-intrinsic inflammasome and its ability to restrict enteric *Salmonella* infections. While previous work compared responses in *Casp11^{-/-}* and *Casp1/11^{-/-}* mice (Knodler *et al.*, 2014, Sellin *et al.*, 2014), here using *Casp1^{-/-}* and *Casp11^{-/-}* mice and enteroids, we unequivocally demonstrate that IECs utilize both inflammatory caspases to launch an intrinsic multilayered innate defense. In this study, we found that both caspase-1 and caspase-11 are required to effectively control an enteric *S*. Typhimurium infection. Caspase-1 dominates the antimicrobial response to *Salmonella* at early time points, while caspase-11 mediated defense plays a larger role later in the course of infection (Figure 3.1A). This timeline agrees with our previous observations that *Casp11^{-/-}* mice carried significantly higher cecal burdens than wildtype mice at 72 h p.i. upon infection with the *S*. Typhimurium $\Delta aroA$ strain (Knodler *et al.*, 2014).

Enteroids offer an attractive alternative to traditional cell culture, providing a physiologically relevant model system derived from the genotypic tissue of interest (Miyoshi and Stappenbeck, 2013). The use of enteroid-derived monolayers enables the study of bacterial-IEC interactions on various genetic backgrounds without the requirement for expression knockdowns prior to infection (Fernando *et al.*, 2017). In murine cecal-derived enteroids, we showed that under baseline conditions IEC-intrinsic caspase-1 plays the major role in restricting intracellular *Salmonella* proliferation (Figure 3.4). In contrast, after pretreatment with IFN- γ to mimic the inflammatory environment that develops during *in vivo* infection, high pathogen loads only

developed in $Casp1/11^{-/-}$ monolayers (Figure 3.8 and 3.9), indicating that both caspase-1 and caspase-11 exert potent antimicrobial responses within immune stimulated IECs.

Studies have previously shown that murine caspase-1 is highly expressed in naïve tissues whereas expression of caspase-11 requires pro-inflammatory induction, potentially in response to activation of the NF- κ B, IRF3 or STAT pathways (Schauvliege *et al.*, 2002, Sellin *et al.*, 2014, Eva *et al.*, 2014). At the beginning of a *S*. Typhimurium infection *in vivo*, baseline tissue expression of *Casp11* is low but is upregulated over the course of infection, whereas *Casp1* transcript levels are high at baseline and decline slightly as the infection progresses (Figure 3.1B) (Sellin *et al.*, 2014). This expression profile is corroborated by our finding that levels of procaspase-11, but not pro-caspase-1, increased in IECs in response to IFN- γ treatment (Figure 3.9A). Our findings suggest that although caspase-1 is sufficient to protect naïve IECs, as the infection proceeds, host inflammatory responses upregulate caspase-11 expression in IECs, leading to increased caspase-11 activity; the combined efforts of these two caspases form a multilayered innate defense that controls intracellular *Salmonella* burdens and protects the host from pathogen attack.

Notably, our *in vivo* mouse studies show that expression of both caspase-1 and caspase-11 is necessary for optimal host defense against *S*. Typhimurium (Figure 3.1). While our enteroid studies also show a clear role for both caspases *in vitro*, expression of either caspase was sufficient to promote inflammasome-mediated control over *Salmonella* expansion in murine IECs, including the expulsion of infected IECs from monolayers (Figure 3.4). These differences likely reflect a role for the inflammatory milieu in fine-tuning IEC inflammasome function as

well as the contribution of inflammatory caspase-mediated activity from other cell types. Caspase-11 requires proinflammatory signaling to be expressed in IECs and macrophages to provide protection against *S*. Typhimurium (Figure 3.9) (Schauvliege *et al.*, 2002, Sellin *et al.*, 2014, Eva *et al.*, 2014). Thus, the impaired IFN- γ expression displayed by infected inflammatory caspase-1 deficient mice may have been insufficient to induce caspase-11 expression to levels sufficient to protect *Casp1^{-/-}* mice (Figure 3.8).

A vital innate defense phenotype mediated by the IEC intrinsic inflammasomes is cell shedding accompanied by pyroptosis (Knodler *et al.*, 2010). Correspondingly, we found that loss of inflammatory caspase signaling in murine enteroids led to decreased cell shedding and resulted in a heavily infected but relatively intact monolayer (Figure 3.4 and 3.9). Defective inflammasome signaling in these monolayers also led to large intracellular microcolonies of Salmonella, as compared to the relatively small intracellular burdens seen in wildtype monolayers. We have previously reported similar findings during S. Typhimurium infection of human IEC lines (Knodler et al., 2014). It is tempting to speculate these increased bacterial burdens are due to the inability of $Casp 1/11^{-/-}$ monolayers to expel infected IECs via pyroptosis. However, in macrophages, there is also an inflammasome-mediated restriction of Salmonella intracellular growth that occurs prior to cell lysis, that is both caspase-1 and caspase-11 dependent, but independent of Gsdmd (Thurston et al., 2016). The identity(s) of the cytosolic antimicrobial agent(s) mediating this protective effect remains unknown, but based on our study design, we cannot rule out its contribution to overall inflammasome-dependent intracellular Salmonella restriction. Interestingly, a low level of cell shedding was observed at 10 h p.i. in $Casp 1/11^{-/-}$ monolayers suggesting there is a minor inflammatory caspase-independent IEC

expulsion component in our enteroid infection model. Previous work found that treatment of $Casp1^{-/-}$ ileal enteroids with FlaTox (a cytoplasmic delivery reagent of Naip5 ligand) induced IEC expulsion that was independent of plasma membrane disruption (Rauch *et al.*, 2017). Through the use of murine knockout and inducible expression models, it was demonstrated that caspase-8, in the absence of caspase-1, can induce IEC cell expulsion in an Asc-dependent manner (Rauch *et al.*, 2017). Thus, although the limited number of shed IECs we noted in the *S*. Typhimurium-infected *Casp1/11^{-/-}* monolayers did not exhibit caspase-1/11 activity (Figure 3.7), they may have been extruded through this caspase-8 dependent mechanism.

We also observed a significant increase in the number of shedding IECs in wildtype and $Casp1^{-/-}$ enteroid-derived monolayers upon IFN- γ pretreatment (Figure 3.9B and C). We hypothesize this increase is due to IFN- γ increasing caspase-11 expression since no significant changes in cell shedding were demonstrated by $Casp11^{-/-}$ or $Casp1/11^{-/-}$ monolayers upon IFN- γ pretreatment (Figure 3.9B and C). However, IFN- γ signaling is complex and induces the expression of a plethora of proinflammatory genes which could impact a caspase-11 driven phenotype. The guanylate-binding proteins (GBPs), for example, are induced upon IFN- γ exposure and can control macrophage antibacterial immune responses in inflammasome-dependent and -independent manners (Wallet *et al.*, 2017). Moreover, they potentiate caspase-11 pyroptosis through the disruption of pathogen-containing vacuoles in macrophages and are known to be expressed in IECs (Meunier *et al.*, 2014, Pilla *et al.*, 2014, Santos *et al.*, 2018, Ingram *et al.*, 2018).

Interestingly, the two inflammatory caspases are required for the rapid induction of IFN- γ in the gut during the early stages of S. Typhimurium infection (12 h p.i.) (Winter et al., 2009). However, the impact of IFN- γ on pathogen burdens and mucosal inflammation is not evident until 48 h p.i. (Songhet et al., 2011, Dolowschiak et al., 2016, Spees et al., 2014). Acute sources of IFN-y include innate lymphoid cells, NK cells, neutrophils and intestinal intraepithelial lymphocytes (Godinez et al., 2008, Klose et al., 2013, Songhet et al., 2011, Dolowschiak et al., 2016, Spees et al., 2014, Hoytema van Konijnenburg et al., 2017). Interestingly, Songhet et al. observed that control over S. Typhimurium burdens within IECs was IFN- γ dependent, and although bone marrow-derived IFN- γR signaling controlled systemic spread, stromal IFN- γR expression was required for this control in IECs (Songhet et al., 2011). Based on our findings, it appears that caspase-11 plays a larger role during the later stages of an acute murine infection, when its expression is induced by proinflammatory signals such as IFN- γ (Figure 3.1 and 3.8). To study the contribution of IEC-intrinsic caspase-11 to overall host defense and the interplay of IFN- γ signaling, further infection studies using cell culture, enteroids or less virulent Salmonella colitis models (e.g. $\Delta aroA$) will be required (Chatfield *et al.*, 1992).

In conclusion, our study defines the importance of the IEC-intrinsic inflammasomes in early host restriction of a *S*. Typhimurium infection. We hypothesize that caspase-1 drives the initial inflammasome mediated antimicrobial response through Nlrc4 mediated detection of Naip ligands; *Salmonella* pathogenicity island-1 (SPI-1) type III secretion system (T3SS) needle components (PrgJ and PrgI) and flagella (FliC) (Mariathasan *et al.*, 2004, Miao *et al.*, 2006, Franchi *et al.*, 2006, Sellin *et al.*, 2014). Upon inflammatory caspase activation, pyroptosis and cell extrusion is initiated, expelling the compromised IEC into the intestinal lumen. By

sacrificing these infected cells, the epithelium maintains its sterility, disables the ability of *Salmonella* to expand their infective niche, meanwhile secreting cytokines and chemokines to recruit professional immune cells to further bolster host defenses. These proinflammatory pathways in turn induce *Casp11* expression and newly primed IECs can now more effectively detect intracellular *Salmonella* through caspase-11 LPS recognition as well as identify those bacteria which have evaded Naip detection through downregulation of SPI-1 and/or *fliC* expression (Crowley *et al.*, 2016). These actions highlight the complex and critical role played by the intestinal epithelium in dealing with invasive bacterial pathogens, such as *Salmonella enterica*.

3.4 Methods

3.4.1 Mice strains and infections

Casp11^{-/-} and *Casp1/11^{-/-}* (*Ice^{-/-}* or *Casp1^{-/-} Casp11^{null/null}*) mice were obtained from Genentech (Kayagaki *et al.*, 2011). *Casp1^{-/-}* mice have been described previously (Rauch *et al.*, 2017). Female C57BL/6 (wildtype) and the various inflammatory caspase-deficient mice were used at 8–12 weeks old and bred under specific pathogen-free conditions at the BC Children's Hospital Research Institute. For oral infections, mice were gavaged with streptomycin (100 mg/kg) 24 h before infection, then orally gavaged with an overnight LB culture of wildtype *Salmonella enterica* serovar Typhimurium SL1344 (naturally streptomycin resistant strain, (Hoiseth and Stocker, 1981)) diluted in PBS (~3 × 10⁶ CFU) and euthanized at 18 h or 72 h p.i. All mouse experiments were performed according to protocols approved by the University of British Columbia's Animal Care Committee and in direct accordance with the Canadian Council on Animal Care (CCAC) guidelines.

3.4.2 Tissue collection and bacterial counts

Mice were anesthetized with isoflurane and euthanized via cervical dislocation. For *Salmonella* enumeration, the cecum, colon and combined cecal and colonic luminal contents were collected and homogenized separately in 1 mL of sterile PBS. Samples were serially diluted and plated onto streptomycin-supplemented LB agar plates and incubated at 37°C overnight. Colonies were then enumerated and normalized to tissue weights. Tissue samples for histology and immunostaining were fixed in 10% neutral buffered formalin (Fischer Scientific) overnight then transferred to 70% ethanol. All fixed tissue was embedded in paraffin and cut into 5 µm sections.

3.4.3 Immunofluorescent staining of tissue

Immunofluorescent staining proceeded as outlined previously (Knodler *et al.*, 2014). In brief, paraffin embedded tissues were deparaffinized by heating to 60°C for 15 min, cleared with xylene, and rehydrated through an ethanol gradient to water. Antigen retrieval was performed in steam heated citrate buffer for 30mins, before cooling to room temperature and washing with water. Tissues were treated in PBS, 0.1% Triton X-100 and 0.05% Tween 20 for 15 mins, then blocked with 5% donkey serum in PBS, 0.01% Triton X-100 and 0.05% Tween 20. Primary antibodies used were Difco *Salmonella* O antisera Group B (Factors 1, 4, 12, 27) (1:1000, BD) and anti-E-cadherin (1:100; BD Biosciences). Tissues were then probed with Alexa Fluor 488-conjugated donkey anti-goat IgG (1:1000; Life Technologies) and Alexa Fluor 568-conjugated donkey anti-rabbit IgG (1:2000; Life Technologies). Tissues were mounted using ProLong Gold Antifade reagent (Life Technologies) containing DAPI for DNA staining. Sections were viewed

on a Zeiss AxioImager microscope and images taken using an AxioCam HRm camera operating through AxioVision software.

3.4.4 Intracellular Salmonella quantification in vivo

Cecal sections that were immunostained for *Salmonella*, E-cadherin and DAPI, were blinded and manually studied at a magnification of ×400 to enumerate infected IECs per crypt, the number of intracellular *Salmonella* in IECs, and the presence of IEC(s) apically shedding from infected crypts (a score of 1 was given when shed IEC(s) were present, while 0 was awarded when no adjacent shedding IEC was present). For all enumerations, five separate cross sections from each mouse background were used, from two or more independent experiments and ten non-adjacent crypts for each cross section were selected. Epithelial integrity for the entire cross section were also evaluated as described by Barthel *et al.* with modification (Barthel *et al.*, 2003, Bhinder *et al.*, 2014) (0, no pathological changes detectable; 1, epithelial desquamation [a few cells shed, surface rippled]; 2, erosion of epithelial surface [epithelial surface rippled, damaged]; 3, epithelial surface severely disrupted/damaged, large amounts of cell shedding).

3.4.5 Generation of cecal enteroids

Enteroids from murine ceca were isolated from each mouse background as previously described (Sato *et al.*, 2009, Fernando *et al.*, 2017, Miyoshi and Stappenbeck, 2013). In brief, the ceca were excised, the tip and base removed, laterally opened to expose the apical surface, while luminal contents were removed and placed in Advanced DMEM/F12 (Gibco) supplemented with Pen Strep (100 U/ml, Gibco) and gentamicin (50 μ g/ml, Gibco) on ice. The tissue was washed ten times in ice-cold Advanced DMEM/F12 (Gibco) with extensive vortexing, then transferred to

Cell Recovery Solution (Corning) and incubated on ice for 30 mins. Under sterile conditions, forceps were used to gently liberate cecal crypts from the underlying tissue and the remaining tissue was discarded. The solution containing the cecal crypts was then centrifuged and washed twice with base media (Advanced DMEM/F12, Gibco) supplemented with Pen Strep (100 U/ml, Gibco), GlutaMAX (1X, Gibco) and HEPES (0.01 M, Gibco)) then diluted 1:1 in Matrigel (Corning). This was pipetted into several 'domes' on a 24-well plate and incubated at 37°C with 5% CO_2 . After the Matrigel solidified, growth media (base media supplemented with 1X condition media from L-WRN cells (CRL-3276, ATCC), N2 (Invitrogen), B27 (Invitrogen), Nacetylcystine (Sigma-Aldrich), nicotinamide (Sigma), mEGF (Invitrogen), A 83-01 (Tocris), SB 202190 (Sigma-Aldrich), and Y-27632 (Abmole)) was added to the well and incubated at 37°C with 5% CO₂ (Miyoshi and Stappenbeck, 2013). L-WRN cells were cultured as previously described and condition media collected every 48h (Miyoshi and Stappenbeck, 2013). Media was changed every three days (growth media without Y-27632 supplementation) and the enteroids were passaged every five to seven days. For IFNy-treated enteroids, growth media was supplemented with murine IFNy (10 ng/mL; Peprotech) or corresponding volume of growth media for 16 h.

3.4.6 Enteroid monolayer seeding and *Salmonella* infection of monolayers

Monolayers were generated as outlined previously (Moon *et al.*, 2014, Fernando *et al.*, 2017) with modifications. First, the growth media was removed, then four Matrigel domes were pooled and disrupted through the addition of ice-cold Cell Recovery Solution and incubation on ice for 30 mins. Enteroids were then centrifuged and washed twice with base media, resuspended in Trypsin-EDTA (0.05%, Gibco) and incubated at 37°C with 5% CO₂ for 10 mins. Enteroids were

then mechanically disrupted into single cell suspensions with repeated pipetting through a p200 tip, and an equal volume of monolayer media (base media supplemented with N2 (Invitrogen), B27 (Invitrogen), and Y-27632 (Abmole) was added. Cells were centrifuged, then resuspended in monolayer media and added dropwise to Geltrex (Gibco) coated coverslips in 24-well plates. Monolayers were incubated at 37°C with 5% CO₂ and media changed 24 h after seeding. Confluent monolayers were infected 72 h after seeding.

S. Typhimurium SL1344 wildtype *glmS::Ptrc-mCherryST* (Knodler *et al.*, 2014) was grown overnight in LB (5g/L NaCl) at 37°C then diluted 1:300 into 10 mL of LB (Miller; 10g/L NaCl) and grown for 4 h at 37°C with shaking (Knodler *et al.*, 2010). The culture was then centrifuged, washed in PBS then diluted in infection media (monolayer media without Pen Strep) at a MOI of 1:50. *Salmonella* containing media was added to the monolayers and incubated at 37°C with 5% CO₂ for 10 mins, then washed three times with PBS, and fresh infection media added for 20 mins. Monolayers were then treated with 50 µg/mL of gentamicin for 40 mins at 37°C with 5% CO₂. Media was discarded then fresh infection media supplemented with 10 µg/mL of gentamicin added and monolayers incubated at 37°C with 5% CO₂ for a total infection period of 10 h. After infection, two 50 µl aliquots of media from each condition were transferred to a black bottom 96-well plate for LDH activity quantification through the CytoTox-ONE Homogeneous Membrane Integrity Assay (Promega) performed according to manufacturer's instructions. Monolayers were washed three times with PBS, fixed in 4% paraformaldehyde (PFA, Thermo Scientific) in the dark at RT for 30 mins, then used for immunostaining.

3.4.7 Immunofluorescent staining of enteroid monolayers

PFA-fixed enteroids on coverslips were treated in PBS, 0.1% Triton X-100 and 0.05% Tween 20 for 15 mins, then blocked with 2% donkey serum in PBS, 0.01% Triton X-100 and 0.05% Tween 20 overnight. Coverslips were then stained with Alexa Fluor 488-phalloidin (1:2000; Life Technologies) for 30 mins, washed and mounted using ProLong Gold Antifade reagent (Life Technologies) containing 4',6-diamidino-2-phenylindole (DAPI) for DNA staining. For determination of inflammatory caspase activity, the staining proceeded as outlined by Knodler *et al.* (Knodler *et al.*, 2010). One hour prior to the end of infection, FAM-YVAD-FMK (Immunochemistry Technologies) was diluted 1:30 into infection media and incubated at 37°C with 5% CO₂ for 1 h, and further prepared according to manufacturer's instructions, before incubation with Alexa Fluor 488-phalloidin and mounted using ProLong Gold Antifade reagent. Sections were viewed on a Zeiss AxioImager microscope and images taken using an AxioCam HRm camera operating through AxioVision software.

3.4.8 Cell shedding, infection and intracellular *Salmonella* quantification of enteroid monolayers

Immunostained enteroid monolayers images were obtained at a magnification of ×200 (shedding) or ×400 (infection/intracellular *Salmonella*) then scorer's blinded and images evaluated using ImageJ (version 1.52i). Shed IECs were defined as high intensity DAPI signals (signals present after gating minimal threshold >200) while intact IECs were defined as lower intensity DAPI signals (signals present after gating minimal threshold >30). Shed and total IECs were enumerated through ImageJ 'Analyzed Particles' (>125inch² pixel units; 0.10-1.00 circularity). Infected IECs and intracellular *Salmonella* were enumerated by eye. For all quantifications at least four images per condition were evaluated from two or more independent experiments.

3.4.9 Western blotting

Cell lysates were prepared as outlined previously (Knodler *et al.*, 2014). Enteroids were resuspended in RIPA buffer with cOmplete protease inhibitors (Roche), sonicated, then centrifuged at 16,000xg for 20 min at 4°C. Total protein was estimated (660nm Protein Assay; Pierce) and 10µg of whole cell lysate prepared according to manufacturer's instructions in 1X Bolt LDS Sample Buffer with 1X Bolt Reducing Agent (Life Technologies) and heated at 70°C for 10 min. Proteins were separated by Bolt 12% Bis-Tris Gel (Life Technologies), transferred to PVDF membrane (Life Technologies), followed by immunoblotting with mouse monoclonal anti-caspase-11 (p20 Flamy-1;1:1000; AdipoGen), mouse monoclonal anti-caspase-1 (p20 Casper-1;1:2000; AdipoGen), or mouse monoclonal anti- β -actin (G043; 1:2000; Applied Biological Materials), then with horse α -mouse IgG:HRP (7076; 1:2000; Cell Signaling Technologies).

3.4.10 Enzyme-linked immunosorbent assay (ELISA)

Mice were infected as described above, 0.5-1 cm of the cecum excised, washed extensively in PBS then stored on ice in *ex vivo* secretion medium (FBS (2%, Sigma-Aldrich), RPMI (Gibco), Pen Strep (100 U/ml, Gibco), Sodium Pyruvate (1mM, Gibco), MEM non-essential amino acids (1X, Sigma-Aldrich), gentamicin (100 μ g/mL, Gibco)). Streptomycin pretreated control ceca were collected from wildtype and *Casp1/11^{-/-}*. Ceca and secretion medium were transferred under sterile conditions to a 24-well plate for 24h incubation. Media was then collected and

centrifuged at 4C, supernatant collected and stored at -80C. Protein concentration was estimated as described above and 17 μ g of total protein probed per a well in duplicate performed according to the manufacturer's instructions (murine IFN- γ ELISA MAXTM Deluxe Set; BioLegend).

3.4.11 Statistical analysis

All results presented in this study are expressed as the mean values \pm standard errors (SEM). Mann-Whitney U-test (to evaluate nonparametric data), student *t*-test and one-way ANOVA (to evaluate parametric data) were performed using GraphPad Prism software, version 7.02 for Windows. A p-value of 0.05 or less was considered significant, with asterisks denoting significance in figures. **Chapter 4:** Inflammasome activation coordinates early intestinal mucosal defense against the enteric pathogen *Salmonella*

4.1 Introduction

Inflammasomes are macromolecular cytoplasmic signaling complexes that activate the inflammatory caspases (caspase-1 and -11 in mice; -1, -4 and -5 in human), leading to the processing of the pro-inflammatory cytokines, interleukin-1β (IL-1β) and IL-18 as well as the induction of a specialized form of inflammatory cell death termed pyroptosis. Since the initial characterization of the inflammasome in 2002, major strides have been made in defining its functional importance in classic immune and inflammatory cell types (Martinon *et al.*, 2002). However, expression of inflammasome components is not limited to myeloid cells but also of functional importance to other cell lineages such as keratinocytes, endothelial cells, fibroblasts and intestinal epithelial cells (Sand *et al.*, 2018, Cheng *et al.*, 2017, Kelly *et al.*, 2019, Knodler *et al.*, 2014, Sellin *et al.*, 2014).We and others have observed the expression of active inflammatory caspases in various epithelial cell lines and showed the inflammasome plays a vital role in innate defense against invading bacterial and viral pathogens in the intestinal mucosa (Shi *et al.*, 2014, Knodler *et al.*, 2014, Sellin *et al.*, 2014, Birchenough *et al.*, 2016, Zhu *et al.*, 2017).

The intestinal epithelium provides both a structural and biochemical barrier, physically sequestering bacteria and other noxious stimuli within the gut lumen and away from the mucosal surface through the generation of a mucus barrier (Allaire *et al.*, 2018, Allaire *et al.*, 2017). Moreover, the epithelium can actively defend the host when challenged by pathogens through the release of antimicrobial factors (Allaire *et al.*, 2018). Our group and others previously reported

that intestinal infection by the enteric pathogen Salmonella enterica serovar Typhimurium (S. Typhimurium) was restricted from expansion and systemic spread through an IEC-intrinsic defense mechanism (Knodler et al., 2014, Sellin et al., 2014). S. Typhimurium is a clinically important invasive bacterial pathogen that causes self-limiting gastroenteritis in humans as well as in mice pretreated with antibiotics to deplete competing commensal microbes (Crowley et al., 2016, LaRock et al., 2015). During its infection, a population of the S. Typhimurium that has invaded the intestinal epithelium escapes from the S. Typhimurium containing vacuole (SCV) and into the cytosol of these cells. While this pathogenic strategy can lead to uncontrolled intracellular pathogen replication, it also activates the epithelial-intrinsic inflammasome. This triggers the extrusion of infected cells into the gut lumen, ultimately limiting S. Typhimurium proliferation within the epithelium as well as preventing the rapid systemic spread of the infection (Knodler et al., 2010, Knodler et al., 2014, Sellin et al., 2014, Chapter 3). Notably, luminal numbers of S. Typhimurium were also significantly increased during infection of inflammasome deficient mice, raising the question of whether other inflammasome-dependent defense mechanisms, aside from epithelial cell sloughing, are triggered during infection (Chapter 3). Therefore, further work characterizing the role that the intestinal inflammasome plays in overall mucosal host defense is sorely needed.

Several studies have implicated inflammasome signaling in controlling goblet cell release of mucins in response to enteric bacteria (Birchenough *et al.*, 2016, Wlodarska *et al.*, 2014, Nishida *et al.*, 2009, Nowarski *et al.*, 2015, Pu *et al.*, 2019). Interestingly, Birchenough and colleagues demonstrated through the use of colonic explants and *in vitro* stimulation assays, a new 'sentinel' subtype of goblet cells (Birchenough *et al.*, 2016). These specialized goblet cells, located at the

apical tip of colonic crypts, rely upon inflammasome signaling to induce crypt-wide mucin release upon cellular detection of microbial products, but this response is IL-18 independent. While an intriguing finding, it still remains unclear how these cells respond *in vivo* during the course of an enteric infection and if inflammasome activation independent of sentinel goblet cells affects mucin secretion and mucus barrier function.

IL-18 has also been described to play a role in goblet cell development and secretion (Nowarski et al., 2015, Ratsimandresy et al., 2017, Nishida et al., 2009). Epithelial IL-18 signaling was correlated with reduced goblet cell maturation and increased goblet cell loss in a dextran sulphate sodium (DSS) model of colitis, and an *Il-18* deficient mouse presented with significantly increased goblet cells and Muc2 expression (Nowarski et al., 2015, Nishida et al., 2009). IL-18 can induce these goblet cell phenotypes both directly and indirectly through IL-22 signaling (Nowarski et al., 2015, Ratsimandresy et al., 2017). IL-22 is a cytokine vital for mucosal host defense. It triggers epithelial cell proliferation, induces antimicrobial protein expression and release as well as increased mucin production and secretion (Rutz et al., 2013). IL-18 can promote IL-22 production directly or indirectly, by potentiating IL-23 signaling, as well as driving the proliferation and maintenance of type 3 innate lymphoid cells (ILC3), the major cellular source of IL-22 in the intestinal mucosa (Dudakov et al., 2015, Victor et al., 2017, Munoz et al., 2015, Mattner et al., 2005). Significant work has gone into elucidating the IL-22 signaling pathway, however its potential interactions with inflammatory caspases, especially in the context of an acute S. Typhimurium infection have not been explored.

To address these questions, we infected inflammatory caspase-deficient mice (*Casp1/11^{-/-}*) with *S*. Typhimurium and measured their early mucosal responses (i.e. at 18h post infection (p.i.)) as compared to wildtype mice. We found that loss of functional inflammasome signaling significantly increased intestinal *S*. Typhimurium burdens in mice at early time points, along with a decrease in cecal pathology scoring, particularly with respect to goblet cell loss. In addition to increased *S*. Typhimurium intracellular burdens as previously reported, we observed that loss of inflammatory caspases led to defects in mucin barrier thickness, as well as decreased antimicrobial lectin and IL-22 production. When we targeted IL-22 production in wildtype mice through the delivery of an IL-22 neutralizing antibody, these mice presented with increased pathogen burdens, decreased mucus thickness and Reg3 β/γ production similar to that observed in *Casp1/11^{-/-}* mice. Thus, the inflammasome induces IL-22 signaling to coordinate multiple layers of innate defense at the gut mucosal surface that ultimately restricts enteric pathogen infections and prevent their systemic spread.

4.2 Results

4.2.1 *Casp1/11^{-/-}* mice carry higher intestinal *Salmonella* burdens compared to *Casp1/11^{+/+}* and *Casp1/11^{+/-}* littermate controls

To test the effect of inflammatory caspases in host defense against an enteric bacterial infection, we employed an acute 18h *S*. Typhimurium infection. Double-deficient $Casp1/11^{-/-}$ mice were backcrossed onto the C57BL/6 (C57BL/6NCrl) background, and the resulting heterozygotes bred to generate $Casp1/11^{+/+}$, $Casp1/11^{+/-}$ and $Casp1/11^{-/-}$. In the murine genome, Casp1 and Casp11 are located approximately 1,500 base pairs apart, which is too close to be segregated by recombination (Kayagaki *et al.*, 2011). Therefore, mice were genotyped based on their *Casp1*

mutation status and periodically screened to ensure they also were $Casp11^{null}$, (out of all mice screened, there were no instances where only one mutation was present). The resulting $Casp1/11^{+/+}$, $Casp1/11^{+/-}$ and $Casp1/11^{-/-}$ littermates were co-housed in the same cages to reduce any potential cage dependent effects due to differences in the gut microbiota.

Casp1/11^{-/-} mice proved highly susceptible to *S*. Typhimurium infection, carrying heavy cecal pathogen burdens compared to both *Casp1/11^{+/+}* and *Casp1/11^{+/-}* (** P<0.01; Figure 4.1A). It appears only one functional copy of *Casp1* and *Casp11* is required for mucosal protection against *S*. Typhimurium because there was no significant difference in *S*. Typhimurium loads for the cecum, colon or intestinal luminal contents between *Casp1/11^{+/+}* and *Casp1/11^{+/-}* (Figure 4.1A). At 18h p.i., there was minimal systemic spread into the liver and spleen (Figure 4.1B), however there were higher burdens in the mesenteric lymph nodes of *Casp1/11^{-/-}* (* P<0.05; Figure 4.1B). The cecal histopathology of *Casp1/11^{+/+}* and *Casp1/11^{+/-}* also appeared similar, with both genotypes presenting with severely damaged epithelial surfaces and large numbers of sloughed epithelial cells found in the lumen (Figure 4.1C, inset panels). This was markedly different from *Casp1/11^{-/-}* ceca, that largely maintained their epithelia (Figure 4.1C). Thus, functional inflammasome signaling is required for intestinal protection against an acute *S*. Typhimurium infection and is not an artifact introduced through parental source or separate cage housing.



Figure 4.1 Functional caspase-1 and -11 are required for the restriction of an acute enteric S. Typhimurium infection *in vivo*.

Streptomycin-pretreated $Casp1/11^{+/+}$, $Casp1/11^{+/-}$ and $Casp1/11^{-/-}$ mice were orally infected with SL1344 S. Typhimurium (3 × 10⁶ CFU), and (A) intestinal tissues and luminal contents and (B) systemic tissues were collected at 18h post infection (p.i.), plated and bacterial numbers enumerated. Each symbol represents one animal. Mean and SEM are indicated. Results are from ≥3 independent experiments. Statistical significance calculated with Mann-Whitney U-test; n.s. (not significant) P > 0.05; *P < 0.05; *P < 0.01. (C) Representative H&E staining of cecal tissue. Original magnification ×100, Inset ×200; scale bars 50µm.

4.2.2 Salmonella infected wildtype mice develop exaggerated cecitis compared to

inflammasome deficient mice

C57BL/6 (wildtype), single inflammatory caspase knockout mice ($Casp1^{-/-}$, $Casp11^{-/-}$) and

Casp1/11^{-/-} were orally gavaged with *S*. Typhimurium or PBS (Ctrl) and cecal histopathology

was evaluated at 18h p.i. by three blinded observers. S. Typhimurium infection increased cecal

histopathology in all backgrounds as compared to PBS treatment (Figure 4.2A and B). However,
wildtype mice displayed a significantly greater pathology score (median total = 14, out of a possible 17) than the inflammatory caspase-deficient mice (Figure 4.2A and B), represented by increased scores in all categories (submucosal edema, epithelial crypt hyperplasia, goblet cell depletion, epithelial integrity, as well as mononuclear and polymorphonuclear leukocyte infiltration in both the submucosa and mucosa). The most pronounced differences were observed in epithelial integrity and goblet cell depletion (Figure 4.2B). The ceca of Casp1^{-/-}, Casp11^{-/-} and $Casp 1/11^{-/-}$ mice presented with a largely intact cecal epithelium, with crypt structures largely maintained throughout all cecal cross-sections (Figure 4.2A). Some sloughed epithelial cells were still evident in the infected ceca of inflammatory caspase-deficient mice; however the epithelial surface of wildtype mice was severely damaged presenting with large areas of sloughing cells and the occasional complete loss of crypt architecture (Figure 4.2A). The crypts of wildtype mice were also characterized by overt goblet cell depletion, with an almost complete loss of mucin-filled goblet cells in their cecal mucosa. In contrast, large, mucin-filled goblet cells could be observed throughout the crypts of the inflammatory caspase-deficient mice, especially at their apical tips (Figure 4.2A, inset panels). Goblet cell depletion is a manifestation of goblet cell hyper-secretion, wherein goblet cells release their contents into the gut lumen. While this response increases the thickness of the intestinal mucus layer, it also makes the goblet cells difficult to observe via H&E staining, since they no longer present with their characteristic purple "full" goblet morphology. These findings confirm that goblet cells of inflammatory caspase-deficient mice are impaired in their ability to respond to pathogenic stimuli and demonstrates this phenotype extends to an acute in vivo S. Typhimurium infection (Birchenough et al., 2016, Wlodarska et al., 2014).





Figure 4.2 Wildtype mice suffer accelerated tissue damage during a *S*. Typhimurium infection compared to inflammatory caspase knockout mice.

(A) Representative H&E staining of cecal tissue from streptomycin-pretreated wildtype, $Casp1/11^{-/-}$, $Casp1^{-/-}$ and $Casp11^{-/-}$ mice 18h after PBS treatment (Ctrl); or 18h p.i. with *S*. Typhimurium. Original magnification ×100; Inset ×200; scale bars 150 µm (inset 50 µm). (B) Comparative cecal histological damage scores of mice given PBS treatment (Ctrl) or infected with *S*. Typhimurium for 18h. Bars represent the damage scores from ≥3 independent experiments with at least n=6 per a genotype. Means are indicated. Statistical significance calculated with Mann-Whitney U-test; n.s. P > 0.05; *P < 0.05; *P < 0.01; ***P < 0.001.

4.2.3 Inflammatory caspase-deficient mice display altered cecal epithelial glycosylation

staining and decreased colonic mucin barrier thickness compared to wildtype mice

The intestinal epithelium is separated from the luminal contents by the mucus layer, which serves as a molecular sieve to prevent most bacteria from reaching the apical epithelial surface (Johansson and Hansson, 2016). In the cecum, the mucus barrier exists as a single layer, loosely associated with the epithelium while in the colon, mucus forms a double layer with the inner mucus layer firmly attached to the epithelium. To uncover if the mucus layer was altered during an acute S. Typhimurium infection, MethaCarn-fixed cecal tissue sections were immunostained for *Ulex europaeus* agglutinin I (UEA-1; green), a lectin which preferentially binds glycans containing α -linked fucose residues, and Muc2 (red), the major mucin protein present in the murine intestinal mucus layer (Figure 4.3A). UEA-1 staining of wildtype cecal tissues revealed vivid staining for fucosylated moieties in goblet cells at the base of cecal crypts as well as increased fucosylation of the surrounding epithelial cells (Figure 4.3A). In contrast, $Casp 1^{-/-}$ and $Casp 1/11^{-/-}$ mice demonstrated only minimal UEA-1 staining that was limited to the apical epithelial surface, while the ceca of $Casp11^{-/-}$ presented with comparatively increased UEA-1 positive signals at the base of cecal crypts, but not to the same intensity observed in wildtype tissues (Figure 4.3A).



Figure 4.3 An acute *S*. Typhimurium infection alters cecal epithelial glycosylation and increases mucin layer width through inflammasome dependent mechanisms.

Streptomycin-pretreated wildtype, $Casp1/11^{-/-}$, $Casp1^{-/-}$ and $Casp11^{-/-}$ mice were orally infected with *S*. Typhimurium for 18h (A) Representative cecal immunostaining for fucosylation (UEA-1; green), Muc2 (red) and DAPI (blue). (B) Representative colonic immunostaining for E-cadherin (green), Muc2 (red) and DAPI (blue). Original magnification ×200; scale bars 100µm. (C) Mean colonic mucin layer thickness 18h after PBS treatment (Ctrl) or *S*. Typhimurium infection from n = 5 mice and at least 2 independent experiments. Mean and SEM indicated. Statistical significance calculated with student t-test n.s. P > 0.05; **P < 0.01; ***P < 0.001.

Staining for Muc2 in cecal tissues from all infected mice revealed 'whispy' Muc2 signals concentrated at the openings of crypts that diffused throughout the entire cecal lumen (Figure 4.3A). In the case of wildtype mice, the majority of their apical goblet cells had either released

their contents or were in the process of releasing their contents (Figure 4.3A) whereas in the inflammasome deficient mice, the Muc2 signal usually presented as "globules" at the epithelial surface or inside intact apical goblet cells. Beyond this analysis, it proved exceedingly difficult to quantify the Muc2 signal because the cecal mucus layer was not well preserved (Figure 4.3A). The streptomycin- *S*. Typhimurium infection model produces very watery contents in the cecal lumen, and without a hard interface, such as a fecal pellet, preservation of the mucus layer is problematic. Therefore, regardless of mouse background, it was difficult to make conclusions about the thickness of the cecal mucus layer thickness.

In contrast, the distal colonic luminal contents remain relatively solid during an acute *S*. Typhimurium infection, allowing better preservation of the colonic mucin layer, therefore colonic MethaCarn-fixed sections were also collected and immunostained for Muc2. A distinct and well-defined mucus layer was present in the colons of all infected mouse genotypes, and at baseline, the widths of all mucus layers were similar (Figure 4.3B and C). Upon *S*. Typhimurium infection, Muc2 staining revealed the colonic mucus layer increased 2.5-fold in wildtype mice, with its thickness averaging 121µm (Figure 4.3C) suggesting significantly increased mucin release by wildtype goblet cells during infection. In contrast, staining for Muc2 on tissues from the inflammatory caspase-deficient mice revealed much less Muc2 staining in their colons (Figure 4.3B and C) with each deficient mouse strain displaying a thinner mucus layer that was not significantly different in width from their baseline levels (Figure 4.3C). Interestingly, in all inflammasome deficient mice, apical goblet cells filled with Muc2 were readily observed at the tips of colonic crypts, indicating these cells were not receiving the necessary signals to induce mucin secretion (Figure 4.3B). In keeping with the thicker mucus layer observe in wildtype mice, their apical goblet cells were virtually absent, with their released contents most likely contributing to the increased mucus layer thickness (Figure 4.3B).



Figure 4.4 Infection induced expression of antimicrobial Reg3 lectins is decreased in *Casp1/11^{-/-}* **mice.** Streptomycin-pretreated wildtype and *Casp1/11^{-/-}* ceca were collected 18h after PBS treatment (Ctrl); or 18h p.i. with *S*. Typhimurium. Relative transcription of (A) Reg3β and (B) Reg3γ by qPCR. Mean and SD indicated. Results are from \geq 3 independent experiments. Statistical significance calculated with student t-test **P < 0.01; ***P < 0.001. (C) Representative immunostaining for RegIII-β (red) and DAPI (blue) of PBS control and 18h p.i. *S*. Typhimurium cecal tissue. Original magnification ×200; scale bars 150 µm.

4.2.4 Inflammatory caspase-deficient mice show decreased antimicrobial lectin expression compared to wildtype mice

The intestinal epithelium secretes a variety of antimicrobial peptides to protect the epithelium from pathogenic attack (Allaire *et al.*, 2018). Thus, to evaluate if the presence of the inflammatory caspases influences intestinal antimicrobial expression, wildtype and *Casp1/11^{-/-}* cecal tissues were examined for differential expression of *Reg3y* and *Reg3β*. There were no significant differences between wildtype and *Casp1/11^{-/-}* transcript levels for both *Reg3* genes in streptomycin pretreated uninfected controls (Figure 4.4A). However, after an 18h *S*. Typhimurium infection, cecal transcripts for *Reg3y* and *Reg3β* were significantly increased in wildtype tissues (90-130 fold) as compared to *Casp1/11^{-/-}* (50-60 fold) (Figure 4.4A and B) (**, P < 0.01 and *, P < 0.05, respectively).

Cecal sections from 18h PBS mock treated (Ctrl) and *S*. Typhimurium infected wildtype, $Casp1^{-/-}$, $Casp11^{-/-}$ and $Casp1/11^{-/-}$ mice were then subjected to immunostaining for Reg3 β . There was minimal signal at baseline for all mouse backgrounds (Figure 4.4C). Upon *S*. Typhimurium infection, cecal tissues from wildtype mice showed strong and widespread Reg3 β immunostaining along the entire length of their cecal crypts, with Reg3 β being primarily expressed by enterocytes, as assessed by their cellular morphology (Figure 4.4C). Cecal tissues from infected inflammatory caspase-deficient mice displayed less overall positive staining, with only a few crypts containing small patches of one to three Reg3 β positive enterocytes (Figure 4.4C). This indicates a functional inflammasome is required for infection-induced upregulation of these antimicrobial C-type lectins. **4.2.5** Infection-induced IL-22 expression and production is decreased in *Casp1/11^{-/-}* mice Interleukin-22 (IL-22) is a unique cytokine that is produced by immune cells but acts primarily on non-hematopoietic cells such as epithelial cells. In the intestine, increased expression of IL-22 is linked to crypt epithelial hyperplasia, mucus secretion as well as C-type lectin production. Notably, these phenotypes show distinct differences between wildtype and inflammatory caspase-deficient mice as we have outlined above. Therefore, expression of *Il-22* was measured in wildtype and *Casp1/11^{-/-}* tissue. There were no significant differences between wildtype and *Casp1/11^{-/-}* in terms of relative transcript levels for IL-22 in streptomycin pretreated uninfected controls (Figure 4.5A). However, after an 18h *S*. Typhimurium infection, transcription of *Il22* dramatically increased over 600-fold in wildtype tissues while only 200-fold in *Casp1/11^{-/-}* mice (Figure 4.5A) (**, P < 0.01), indicating that the distinct mucus and antimicrobial lectin phenotypes observed in this study could be IL-22 mediated.



Figure 4.5 Wildtype mice treated with an anti-IL-22 neutralizing antibody phenocopy increased pathogen burden and decreased epithelial pathology displayed by *Casp1/11^{-/-}* mice.

(A) Relative expression of *II22* in cecal tissues from streptomycin-pretreated wildtype and *Casp1/11^{-/-}*mice 18h after PBS treatment (Ctrl) or *S*. Typhimurium infection measured by qPCR. Each symbol represents one animal. Mean and SEM are indicated. Results are from \geq 3 independent experiments. Streptomycin-pretreated wildtype and *Casp1/11^{-/-}*mice were treated *i.p.* with IL-22 neutralizing antibody (aIL-22) or isotype control (Isotype; IgG), then orally infected with *S*. Typhimurium for 18h. (B) Cecal tissue IL-22 concentrations. Mean and SEM indicated. Statistical significance calculated with student t-test; *P < 0.05; **P < 0.01; ***P < 0.001. (C) Cecal pathogen burdens at 18h p.i. Each symbol represents one animal. Mean and SEM indicated. Results are from \geq 2 independent experiments. Statistical significance calculated with Mann-Whitney U-test; *P < 0.05; **P < 0.01; ***P < 0.001. (D) Representative H&E staining of cecal tissue. Original magnification ×100; Inset ×200; scale bars 200 µm (inset 50 µm).

4.2.6 IL-22 neutralization in wildtype mice increases pathogen burdens and decreases cecal histopathology

To assess if IL-22 was the central mediator for the differential mucosal response between wildtype mice and mice lacking the inflammatory caspases, wildtype and *Casp1/11^{-/-}* mice were treated with either 150µg of IL-22 neutralizing antibody (α IL-22) or IgG isotype control antibody (Isotype; IgG) 1h prior to *S*. Typhimurium delivery. Wildtype mice treated with the isotype control displayed a robust cecal IL-22 response, secreting 2-fold more than *Casp1/11^{-/-}* isotype controls (** P < 0.01; Figure 4.5B). In contrast, α IL-22 administration significantly reduced IL-22 protein levels in both wildtype and *Casp1/11^{-/-}* mice below 70pg/mL (67.3pg/mL and 41.4pg/mL respectively, *** P < 0.001; Figure 4.5B). Interestingly, *Casp1/11^{-/-}* mice did produce IL-22 in their cecal tissues, although at significantly lower levels than wildtype (** P < 0.01; Figure 4.5B), which mirrors the trends seen in the qPCR data (Figure 4.5A).

Isotype control treatment did not impact the higher pathogen numbers found in the ceca of $Casp1/11^{-/-}$ mice (** P < 0.01; Figure 4.5C). However, IL-22 neutralization in wildtype mice significantly increased their cecal *S*. Typhimurium burdens 4.5-fold compared to isotype treated wildtype mice (* P < 0.05; Figure 4.5C). This increase in cecal pathogen loads remained significantly lower than those recovered from IgG or α IL-22 treated *Casp1/11^{-/-}* mice indicating IL-22 neutralization in wildtype mice produces an intermediate colonization phenotype. That impaired IL-22 production contributes, but is not solely responsible for the higher pathogen burdens carried by inflammasome deficient mice (Figure 4.1 and Figure 4.5C). Cecal pathogen burdens were not significantly different between IgG or α IL-22 treated *Casp1/11^{-/-}* mice. This

suggests the low levels of IL-22 produced by $Casp1/11^{-/-}$ mice is insufficient to influence *S*. Typhimurium burdens (Figure 4.5B and C).

In comparing the H&E stained cecal sections of the four groups, the most pronounced histopathological differences observed during infection between the α IL-22 or IgG treated wildtype mice were displayed in the cecal epithelium. Isotype treated wildtype mice presented with a severely damaged epithelium, as well as a high degree of epithelial desquamation and the occasional loss of defined crypt architecture (Figure 4.5D). In contrast, epithelial damage was less pronounced in wildtype mice given α IL-22, as well as in IgG and α IL-22 treated Casp1/11^{-/-} mice (Figure 4.5D). These ceca retained their crypt architecture and while they did display some cell sloughing, it was to a much lesser degree than that displayed by the IgG treated wildtype mice (Figure 4.5D). Strikingly, goblet cells, especially at the apical tips of cecal crypts, were present in aIL-22 treated wildtype mice, whereas they were virtually depleted in IgG treated wildtype mice (Figure 4.5D, inset panels). Regardless of aIL-22 or IgG treatment, the $Casp 1/11^{-/-}$ mice also retained their goblet cells (Figure 4.5D, inset panels), suggesting IL-22 could be a major contributor to the differing histopathological scoring observed between wildtype and inflammatory caspase deficient mice, especially in the category of goblet cell depletion (Figure 4.2B).

4.2.7 IL-22 neutralization in wildtype mice phenocopies the impaired mucus barrier and antimicrobial response of inflammasome deficient mice

MethaCarn-fixed colonic tissue sections from α IL-22 or IgG treated *S*. Typhimurium infected mice were also subjected to immunostaining for Muc2 to evaluate if IL-22 neutralization

impacted their mucosal response. IL-22 neutralization produced markedly thinner colonic mucus layers in infected wildtype mice, as compared to isotype controls (Figure 4.6A). This suggests that α IL-22 treatment prevents mucin release by goblet cells during *S*. Typhimurium infection, leading to mucus layers that are similar in thickness to those observed in the inflammasome deficient mice. Neither IgG nor α IL-22 treatment differentially affected mucus barrier thickness in *S*. Typhimurium infected *Casp1/11^{-/-}* mice (Figure 4.6A), with both treatment groups presenting thin colonic mucus layers similar to α IL-22 treated wildtype (Figure 4.6A).



Figure 4.6 IL-22 neutralization of wildtype mice impairs *S*. Typhimurium induced mucin and antimicrobial response to similar levels as inflammasome deficient mice.

Streptomycin-pretreated wildtype and *Casp1/11^{-/-}* mice were treated *i.p.* with IL-22 neutralizing antibody (α IL-22) or isotype control (Isotype), then orally infected with *S*. Typhimurium for 18h. Representative immunostaining for (A) Muc2 (red), E-cadherin (green) and DAPI (blue). (B) Reg3 β (red) and DAPI (blue). Original magnification ×200; scale bars 150µm.

Cecal sections from infected mice given IL-22 neutralizing antibodies, or the isotype controls were also immunostained for Reg3 β . A stark contrast was observed between the IgG and α IL-22 treated wildtype ceca (Figure 4.6B). Cecal tissues from wildtype mice given the isotype control displayed vivid Reg3 β positive staining whereas virtually no signal was observed after IL-22 neutralization (Figure 4.6B). Similar to the untreated *S*. Typhimurium infected wildtype (Figure 4C), IgG treated wildtype mice showed Reg3 β immunostaining throughout the length of their cecal crypts (Figure 4.6B). Correspondingly, α IL-22 or IgG treatment had little impact on Reg3 β signal in *Casp1/11^{-/-}* mice, with these mice showing little Reg3 β signal irrespective of treatment (Figure 4.6B). Taken together, these results indicate that IL-22 signaling is required for the inflammasome dependent antimicrobial and mucus barrier defense mechanisms that restrict an acute enteric *S*. Typhimurium infection.

4.2.8 *Il18^{-/-}* and *Nlrp6^{-/-}* deficient mice display decreased mucin barrier widths and comparable *S*. Typhimurium loads to wildtype

Previous work connecting inflammasome signaling with mucin secretion has implicated Nlrp6 (a NOD-like protein receptor upstream of inflammatory caspase signaling) and IL-18 (a cytokine processed by caspase-1 and -11) as important factors (Wlodarska *et al.*, 2014, Birchenough *et al.*, 2016, Nowarski *et al.*, 2015). Therefore, *Il18^{-/-}* and *Nlrp6^{-/-}* deficient mice were infected with *S*. Typhimurium and tissues collected at 18h p.i. to test if their mucosal responses were impaired similar to the inflammatory caspase deficient mice described earlier in this Chapter.

Colonic tissues were MethaCarn-fixed and sections immunostained for Muc2. Both $II18^{-/-}$ and $Nlrp6^{-/-}$ displayed thin mucus layers similar to the mucus widths displayed by $Casp1/11^{-/-}$ mice

(Figure 4.7A and 4B). This is consistent with previous reports detailing impaired goblet cell function by $II18^{-/-}$ and $Nlrp6^{-/-}$ mice (Wlodarska *et al.*, 2014, Nowarski *et al.*, 2015). MethaCarn fixed cecal tissues were also stained for UEA-1 and Muc2 to examine if the infection induced fucosylation was impaired in $II18^{-/-}$ and $Nlrp6^{-/-}$ mice to levels comparable to $Casp1/11^{-/-}$ mice (Figure 4.3A). Interestingly, $Nlrp6^{-/-}$ ceca phenocopied wildtype mice (Figure 4.6B and 3A), displaying vivid UEA-1 positive staining at the base of their crypts by goblet cells and at the apical surface of epithelial cells. In contrast, $II18^{-/-}$ mice presented with minimal UEA-1 binding (Figure 4.6B), with their limited positive staining restricted to the apical epithelial surface, similar to the staining pattern exhibited by $Casp1/11^{-/-}$ mice (Figure 4.3A). This differential infection-induced fucosylation staining between $Nlrp6^{-/-}$ and $Casp1/11^{-/-}$ ceca suggests either that $Nlrp6^{-/-}$ impaired goblet cell function is IL-22 independent or that Nlrp6 expression is restricted to the colon and does not play a role in cecal histopathology.

Finally, 18h p.i. *S.* Typhimurium burdens were determined for $II18^{-/-}$ and $Nlrp6^{-/-}$ mice (Figure 4.6C and D) at both systemic and intestinal sites. Neither the systemic or cecal pathogen burdens of $II18^{-/-}$ or $Nlrp6^{-/-}$ mice were significantly different from wildtype (Figure 4.6C). However, both deficient mice displayed significantly higher pathogen loads compared to wildtype mice in both their colonic tissue and luminal contents (Figure 4.6C). The combination of immunostaining and pathogen burdens for $II18^{-/-}$ and $Nlrp6^{-/-}$ mice indicates that loss of neither protein phenocopies both the mucosal composition and increased *S*. Typhimurium loads displayed by $Casp1/11^{-/-}$ mice (Figure 4.3 and 6). However, the decreased colonic mucin thickness and minimal cecal fucosylation staining by $II18^{-/-}$ suggests this cytokine could be playing a major role in the signalling pathway for acute *S*. Typhimurium induced IL-22 secretion, but further work is required to elucidate all contributors.



Figure 4.7 *II18^{-/-}* and *Nlrp6^{-/-}* mice display an abrogated mucin response to *S*. Typhimurium infection however cecal pathogen burdens are not significantly different from wildtype. Streptomycin-pretreated *II18^{-/-}* and *Nlrp6^{-/-}* mice were orally infected with *S*. Typhimurium for 18h. (A) Representative colonic immunostaining for E-cadherin (green), Muc2 (red) and DAPI (blue). (B) Representative cecal immunostaining for Reg3β (red) and DAPI (blue). Original magnification ×200; scale bars 100µm. Original magnification ×200; scale bars 100µm. Pathogen burdens enumerated from (C) intestinal tissues and luminal contents and (D) systemic tissues collected at 18h p.i. Each symbol represents one animal. Mean and SEM are indicated. Results are from \geq 3 independent experiments. Statistical significance calculated with Mann-Whitney Utest; **P < 0.01; ***P < 0.001.

4.3 Discussion

The intestinal mucosa is the site at which *S*. Typhimurium, as well as other enteric pathogens, launch their assault upon their hosts. Understanding the processes by which the hosts' innate immune system rapidly responds to these threats and launches interconnected layers of defense is vital to our understanding of enteric immunity. Herein, we present evidence that caspase-1 and - 11 act as central mediators of the intestinal mucosal response during the early stages of a *S*. Typhimurium infection. Our data establishes the inflammasome as a key initiator of host defense, whose activation cumulates in IL-22 dependent mucin secretion and antimicrobial lectin production that ultimately aids in the restriction of *S*. Typhimurium at the mucosal surface.

The cellular source of IL-22 in our model is currently under investigation. Established sources of IL-22 include ILC3s, $\alpha\beta$ T cells, $\gamma\delta$ T cells, natural killer (NK) T-cells and macrophages (Dudakov *et al.*, 2015). Study of this cytokine is still an emerging field and preliminary work has identified neutrophils, NK cells and fibroblasts as other possible cellular sources (Zindl *et al.*, 2013, Kumar *et al.*, 2013, Ikeuchi *et al.*, 2005). However, due to the cellular fluidity between some of these cell types and the innate difficulties in designing flow panels to identify IL-22 producing cells, further work is required to conclusively define its cellular sources during an acute *S*. Typhimurium infection. In our model, we observed increased IL-22 production at 18h

p.i. which is too early for the host to mount a T cell mediated response to an antigen it has not been previously exposed to (Pennock *et al.*, 2013). This narrows the list of candidates to tissue resident cells (fibroblasts, monocytes, macrophages and ILC3s) and/or infiltrating innate immune cells (NKT, NK and neutrophils). IL-23 is the main stimulator of IL-22 production, however IL-18 (an inflammasome processed cytokine) can directly induce IL-22 production in NKTs and ILC3s as well as drive ILC3 proliferation and maintenance (Dudakov et al., 2015, Victor et al., 2017, Munoz et al., 2015, Mattner et al., 2005). Interestingly, previous reports indicate that during an oral S. Typhimurium infection, *Il23* transcripts remain relatively low in number until 48h p.i., whereas *Il22* gene transcription is induced as early as 12h p.i. and persists at high levels until 48h p.i. (Keestra et al., 2011). Müller and colleagues identified IL-18 as crucial driver for acute mucosal inflammation as of 12h post S. Typhimurium infection (Muller et al., 2016). They observed that IL-18 levels peaked at 18h p.i., whereas IL-18 deficient mice displayed decreased cecal histopathology as compared to wildtype or IL-1 α/β deficient animals (Muller *et al.*, 2016). Administration of recombinant IL-18 has also been shown to preserve colonic epithelial integrity in Casp1/11^{-/-} mice during the dextran sodium sulfate model of colitis (Dupaul-Chicoine et al., 2010). Müller and colleagues attributed the protective effects of IL-18 during S. Typhimurium cecitis to increased NK cell migration and perforin production in mounting gut mucosal inflammation (Muller et al., 2016). We are currently exploring if these migratory NK cells could also be the source of the IL-22 responsible for the mucosal phenotypes observed in our model. Interestingly, we observed that infected ceca from mice treated with α IL-22 displayed preserved epithelial integrity as compared to IgG treated controls (Figure 4.5D). Further experimentation is required to determine if the protection of epithelial integrity exhibited by wildtype mice receiving α IL-22 treatment, is solely IL-22 mediated, as well as identify the cellular source of IL- 22 and if its production is stimulated through epithelial produced IL-18. These phenotypes are further complicated by the presence of IL-22 binding protein (BP) and the IL-18BP. These are soluble receptors that preferentially bind their respective substrates, thereby reducing circulating levels of bioactive IL-22 and IL-18. IL-18 downregulates IL-22BP, suggesting another IL-22 mediated contribution in our model, in which the ratio of IL-22 to IL-22BP is shifted, increasing the IL-22 potentiated effects (Huber *et al.*, 2012). Increased levels of IL-22BP have been observed in *Casp1/11^{-/-}* mice as compared to wildtype mice at baseline and it would be interesting to see if *Il22bp* expression fluctuates in response to increased IL-18 production during an acute *S*. Typhimurium infection (Ratsimandresy *et al.*, 2017, Muller *et al.*, 2016).

Previous work has established that inflammasome signaling is required for some aspects of goblet cell function (Wlodarska *et al.*, 2014, Birchenough *et al.*, 2016).Our results show inflammasome signaling is not required for homeostatic maintenance of the mucus layer (Figure 4.3C). Upon infection however, inflammatory caspase activation drives a massive release of Muc2, thereby increasing the thickness of the intestinal mucus layer and physically pushing pathogens and other noxious stimuli away from the epithelial surface (Figure 4.3B and C). Depending on their crypt axis location, goblet cells appear to differ in their ability to store and secrete mucins as well as respond to various signaling pathways (Johansson, 2012, Schneider *et al.*, 2018). Recently, Birchenough *et al.* identified a subset of colonic goblet cells they labelled 'sentinel goblet cells,' which react to endocytosed toll-like receptor ligands through activation of the Nlrp6 inflammasome in an IL-18 independent manner (Birchenough *et al.*, 2016). This activation triggers the release of Muc2 from the sentinel goblet cells as well as induces bulk Muc2 secretion from adjacent upper crypt goblet cells through intercellular Ca²⁺ gap junction

signalling (Birchenough et al., 2016). Sentinel goblet cells can currently only be identified based on their function as well as their apical location at the luminal face of the epithelium, therefore it is difficult to estimate the contribution of these cells to the mucus barrier phenotype observed in this study. The mechanism by which endocytosed antigenic S. Typhimurium products would cumulate in Nlrp6 inflammasome activation and sentinel goblet cell secretion is also unclear. Birchenough and colleagues discovered that Myd88-dependent Nox/Duox reactive oxygen species synthesis was upstream of Nlrp6 inflammasome activation (Birchenough et al., 2016) in their model system. Lipotechoic acid was recently identified as a Nlrp6 ligand which when bound, promoted both caspase-1 and -11 activation (Hara et al., 2018). However, in a screen of TLR products by Birchenough et al., lipotechoic acid did not induce goblet cell secretion and lipotechoic acid is an antigen derived from Gram positive bacteria, further complicating the mechanism by which a Gram-negative bacterium such as S. Typhimurium could induce sentinel goblet cell secretion (Birchenough et al., 2016). It is also unclear if the function of sentinel goblet cells relies on inflammasome activity intrinsic to the goblet cells themselves or if they respond to products released by inflammasome activation in the epithelial cells that surround them, or in the inflammatory cells found in the underlying mucosa. Clearly, further work is required to tease out the contribution of sentinel goblet cells to our model.

Previous studies have established that the inflammasome plays a role in early intestinal mucosal defense against *S*. Typhimurium through the physical expulsion of infected epithelial cells (Knodler *et al.*, 2014, Sellin *et al.*, 2014, Chapter 3). This first line of mucosal defense is stimulated as early as 6h p.i. when *S*. Typhimurium first invades and then compromises the cytoplasmic sterility of intestinal epithelial cells, thereby activating caspase-1 and -11 (Sellin *et*

al., 2014, Chapter 3). Once the inflammasome is activated, this triggers the processing and secretion of the cytokine IL-18 as well as the expulsion of the infected cell from the epithelium, through an inflammatory caspase dependent mechanism (Knodler et al., 2014, Muller et al., 2016, Chapter 3). We hypothesize that the inflammasome dependent IL-22 signaling mechanism identified in this Chapter is initiated largely from this epithelial response. We have previous established that epithelial-intrinsic inflammasomes produce active IL-18 in response to a S. Typhimurium infection and in this Chapter, we identified an impairment in colonic mucus thickness and cecal epithelial fucosylation in $Il18^{-/-}$ mice 18h post S. Typhimurium infection (Knodler et al., 2014). In addition, Muller and colleagues measured IL-18 levels at 18h post S. Typhimurium infection, in wildtype and $Il18^{-/-}$ mice that had undergone reciprocal bone marrow transfer, and observed 10-fold more IL-18 secreted from the non-haemopoietic compartment (which includes epithelial cells) as compared to the haemopoietic-derived cells (Muller *et al.*, 2016). This previous work in conjunction with the experiments conducted in this Chapter, indicates that IL-18 derived from intestinal epithelial cells is a strong candidate for the inflammasome derived signal initiating the IL-22 dependent response.

The intestinal mucosal cytokines IL-22 and IL-18 are known to be important in goblet cell function (Allaire *et al.*, 2017, Nowarski *et al.*, 2015, Goto *et al.*, 2014). IL-22 increases intestinal mucin levels through the promotion of the various *Muc* transcripts (Sugimoto *et al.*, 2008, Turner *et al.*, 2013). IL-22 is also integral to the amelioration of colitis through mucus production as well as by promoting gut homeostatic recovery after colitic insult through epithelial/goblet cell restitution (Sugimoto *et al.*, 2008). We observed inflammasome dependent, IL-22 mediated increases in colonic mucus barrier thickness after *S*. Typhimurium infection (Figure 4.3B and C,

Figure 4.6A). The inflammasome drives an increase in intestinal IL-22 levels, most likely through the release and subsequent actions of IL-18, and this induces goblet cells to secrete Muc2, thereby increasing the width of the mucus layer. IL-22 also induces changes in epithelial surface glycosylation (Goto *et al.*, 2014). Goto and colleagues observed increased epithelial fucosylation – induced by IL-22, was able to protect mice from overt *S*. Typhimurium cecitis (Goto *et al.*, 2014). In our acute *S*. Typhimurium infection model, increased fucose staining was evident on the apical surface of enterocytes and in goblet cells in the lower cecal crypts of wildtype mice, while this was absent in *Casp1/11^{-/-}* as well as *Il18^{-/-}* mice (Figure 4.3A). This response is likely mediated by IL-22 signaling that is activated through inflammatory caspase induced IL-18 secretion.

Increased IL-18 levels are linked with the breakdown of mucin barrier integrity, through direct transcriptional effects affecting goblet cell maturation and function (Nowarski *et al.*, 2015, Levy *et al.*, 2015, Nishida *et al.*, 2009). Nowarkski and colleagues observed that deletion of the IL-18 regulator IL-18BP, promoted high levels of circulating IL-18 during DSS-colitis, resulting in severe colitis and significant loss of goblet cells (Nowarski *et al.*, 2015). They also reported that IL-18 inhibited goblet cell maturation by reducing transcript levels of *Glfi1*, *Spdef* and *Klf4* (transcriptional factors vital to goblet cell differentiation and maturation), while transcriptional factors vital for secretory cell lineage development such as *Math1* and *Hes1* were not impacted (Nowarski *et al.*, 2015). This effect of IL-18 signaling was mediated directly through the epithelium with the authors observing that their colitis and goblet cell phenotypes were rescued by using mice with an epithelial specific knockdown of the IL-18 receptor (Nowarski *et al.*, 2015). This indicates that IL-18 signaling can directly affect goblet cell function although further

work is required to delineate the relative contributions of IL-18 and IL-22 to goblet cell function. However, the mucin barrier phenotypes observed in our *S*. Typhimurium acute infection model appear to be predominantly IL-22 dependent due to the thin mucin layer observed in our *S*. Typhimurium infected wildtype mice treated with IL-22 neutralizing antibodies (Figure 4.6A). $II18^{-/-}$ mice also present with thin colonic mucus layers as well as decreased fucosylation staining, but their cecal pathogen burdens were not different from wildtype (Figure 4.6). Therefore, further work is required to determine the contribution of IL-18 secretion to the $Casp1/11^{-/-}$ IL-22 mediated mucosal responses observed in this Chapter and if these phenotypes require other cytokines or chemokines to induce IL-22 secretion.

Strikingly, upon *S*. Typhimurium infection, we also see a rapid upregulation in antimicrobial Reg3 lectins produced by epithelial cells (Figure 4). These responses are also dependent on inflammatory caspases, as well as IL-22 signaling (Figure 4.6B) and ultimately lead to an antimicrobial environment within the cecum that potentially acts against both *S*. Typhimurium and the microbiota. Reg3 β and Reg3 γ both bind bacteria through carbohydrate mediated recognition, with Reg3 β binding both Gram-positive and Gram-negative bacteria, while Reg3 γ preferentially binds Gram-positive bacteria (Miki *et al.*, 2012, Cash *et al.*, 2006). *S*. Typhimurium itself is Reg3 β sensitive but only during the lag to mid-log phase of its growth curve, while *S*. Typhimurium at stationary phase (the growth stage most likely representative of its *in vivo* infection) is resistant (Miki *et al.*, 2012). Interestingly, Reg3 β can also promote sustained intestinal colonization of *S*. Typhimurium through its antimicrobial effects on competing members of the microbiota such as the *Bacteroides* spp. and *Clostridiales* spp. (Miki *et al.*, 2017). *S*. Typhimurium is transmitted from host to host through the fecal oral route of infection which requires an estimated 10^8 colony forming units per gram of feces for successful transmission (Lawley and Walker, 2013, Rivera-Chavez *et al.*, 2017). Since the resident commensal microbiota can block successful infection through its ability to promote colonization resistance, *S*. Typhimurium must carve out an intestinal environment favourable for its survival and proliferation. To do this, *S*. Typhimurium employs a variety of strategies to cause changes in the composition of the gut microbiota, making the gut lumen a more attractive niche. These strategies include promoting host responses that cause the suppression of *Clostridia* spp. and proliferation of *Desulfovibrio* sp. to produce the unique conditions required for *S*. Typhimurium to undergo rapid luminal expansion (Rivera-Chavez *et al.*, 2017, Rivera-Chavez *et al.*, 2016, Thiennimitr *et al.*, 2011). At present, it is unclear (and thus requires further study) whether the inflammasome mediated induction of Reg3 β production in the infected cecum increases *S*. Typhimurium persistence or aids in its overall clearance.

Previous work has established that IL-22 can induce Reg3 expression and secretion (Cash *et al.*, 2006, Zindl *et al.*, 2013). However, understanding the signaling cascade between inflammatory caspase cleaved IL-18 and its impact on IL-22 induced antimicrobial phenotypes is still in its infancy. Ratsimandresy *et al.* observed that treatment of colonic enteroids with recombinant IL-18 or IL-22 both increased *Reg3β* and *Reg3γ* expression, however treatment with IL-18 did not increase *Reg3* transcription to the same high degree as IL-22 stimulation (Ratsimandresy *et al.*, 2017). The authors also demonstrated that naïve *Casp1/11^{-/-}* epithelial cell isolates displayed increased *Il22bp* transcripts which significantly decreased shortly after recombinant IL-18 treatment (Ratsimandresy *et al.*, 2017). This suggests that inflammasome based IL-18 processing could also increase the activity of IL-22 by decreasing competing IL-22BP levels. Munoz and

colleagues also observed interplay between IL-18 and IL-22 signalling (Munoz et al., 2015). They showed that *Il18* expression was downregulated in $Il22^{-/-}$ mice while its expression could be restored with injection of recombinant IL-22. Correspondingly, *Il22* expression was downregulated in *Il18^{-/-}* mice, but its transcription could be restored with recombinant IL-18 injections (Munoz et al., 2015). This signaling network is further complicated by preliminary work suggesting that Reg3 lectins can act as signaling molecules themselves. Aden et al. constructed epithelial specific IL-23R deficient mice which demonstrated reduced $Reg3\beta$ and Reg3y expression and surprisingly, impaired mucosal IL-22 induction (Aden *et al.*, 2016). Interestingly, recombinant Reg3ß administration restored IL-22 levels in these mice through the recruitment of neutrophils (Aden et al., 2016). Ratsimandresy and colleagues also observed that incubation of enteroids with recombinant Reg3 β or Reg3 γ induced phosphorylation of the transcription factor Stat3 and Akt (Ratsimandresy et al., 2017). Therefore, to understand this complicated signaling network and how it protects the host and/or ultimately aids in establishing an infective niche for S. Typhimurium requires further study delineating the interplay between IL-18, IL-22 and the Reg3 lectins.

Taken together, this work provides new insights into the vital role that the inflammasome plays in mounting responses that promote enteric mucosal defense during pathogen infections. In particular, we observed that activation of the inflammatory caspases ultimately leads to IL-22 induced antimicrobial secretion and increased mucus barrier thickness. This cytokine driven defense mechanism is most likely triggered after *S*. Typhimurium has activated the epithelialintrinsic inflammasome, representing an immune cell-derived response to reinforce the epithelial response that is already engaged. Therefore, inflammasome signaling coordinates a multilayered innate defense at the intestinal mucosal surface through IL-22 that protects the host during an acute *S*. Typhimurium infection.

4.4 Methods

4.4.1 Mice strains and infections

 $Casp11^{-/-} and Casp1/11^{-/-} (Ice^{-/-} or Casp1^{-/-} Casp11^{null/null}) mice were obtained from Genentech (Kayagaki$ *et al.* $, 2011). Casp1^{-/-} were obtained from Dr. Isabella Rauch (University of California). Casp1/11^{+/+} (wildtype Casp1 and Casp11), Casp1/11^{+/-} (heterozygous Casp1^{+/-} and Casp11^{+/null}) and Casp1/11^{-/-} (Casp1^{-/-} and Casp11^{null/null}) were generated by heterozygous Casp1/11^{+/-} breeders resulting from a cross of Casp1/11^{-/-} with C57BL/6NCrl (Charles River), all resulting litters were genotyped using the following primers (Neo – 5'GAA GGG TGA GAA CAG AGT ACC TAC A 3'; Casp1F – 5'GGT CTT GTC TCT TAT AGG AGA TGG T 3', Casp1R – 5'GGA ATC AAC CCC AAA CAC TGA AGA 3').$

All mice (8–12 weeks old) were bred under specific pathogen-free conditions at the BC Children's Hospital Research Institute (BCCHRI). For oral infections, mice were gavaged with streptomycin (100mg/kg) 24h prior to infection, then orally gavaged with an overnight LB culture of wildtype *S*. Typhimurium SL1344 (Streptomycin resistant strain) diluted in 1/100 in PBS (~ 2.5×10^{6} CFU) and euthanized at 18h post infection (p.i.). For the IL-22 neutralization experiments, mice were injected *i.p.* 1h prior to infection with 150µg/100µL dose of IL-22 (αIL-22; 8e11, Genentech) or the equivalent amount of mouse isotype-matched control antibody (ragweed; 10D9.1E11.1F12; Genentech). All mouse experiments were performed according to protocols approved by the University of British Columbia's Animal Care Committee and in direct accordance with the Canadian Council on Animal Care (CCAC) guidelines.

4.4.2 Tissue collection and bacterial counts

Mice were anesthetized with isoflurane and euthanized via cervical dislocation. For bacterial cell counts, liver, spleen, mesenteric lymph nodes (MLNs), cecum, colon and combined cecal and colonic luminal contents were collected and homogenized separately in 1 ml of sterile PBS. Samples were serially diluted and plated on streptomycin-supplemented LB agar plates and incubated at 37°C overnight. Colonies were then enumerated and the number normalized to the weight of the tissues. Tissue samples for histology and immunostaining were fixed either in 10% neutral buffered formalin (Fischer Scientific) overnight, then transferred to 70% ethanol or MethaCarn (60% dry methanol, 30% chloroform, 10% glacial acetic acid) overnight, then washed with methanol and transferred to 100% ethanol. All fixed tissue was embedded in paraffin, and cut into 5-µm sections.

4.4.3 Histology scoring

Formalin fixed tissue sections were stained with hematoxylin and eosin (H&E) (performed by the BCCHRI Histology Core Lab) and were examined by three blinded observers to assess histological damage. Tissue sections were assessed as outlined in Barthel *et al.* with modification (Barthel *et al.*, 2003, Bhinder *et al.*, 2014). For (i) submucosal edema (0, no change; 1 (the submucosa accounts for <50% of the diameter of the entire intestinal wall [tunica muscularis to epithelium]), mild; 2, moderate (50 to 80%); 3, severe (>80%)); (ii) hyperplasia (0, no change; 1, 1 to 50% increase in crypt length from control slide; 2, 51 to 100%) (iii) goblet cell depletion (0,

goblet cells present throughout the crypt (approximately >7 goblet cells/high-power field); 1, mild depletion (goblet cells missing from ~50% of crypt; approximately 2 to 7 goblet cells/highpower field); 2, severe depletion (virtually absent from crypt; <2 goblet cell/high-power field)); (iv) epithelial integrity (0, no pathological changes detectable; 1, epithelial desquamation (a few cells sloughed, surface rippled); 2, epithelial surface severely disrupted/damaged, large amounts of cell sloughing, patchy crypt architecture (approximately <30% of tissue loss crypt structures); 3, ulceration, complete loss of crypt architecture); (v) mucosal mononuclear cell infiltration (per ×400 magnification field) (0, no change; 1, <20; 2, 20 to 50; 3, >50 cells/field); and (vi) submucosal polymorphonuclear leukocytes and mononuclear cell infiltration (per ×400 magnification field) (0, no change; 1, <20; 2, 21 to 50; 3, >50 cells/field). The maximum possible score was 17. Fleiss' Kappa coefficient was 0.83, indicating almost perfect agreement between scorers.

4.4.4 Immunofluorescent staining

Immunofluorescent staining proceeded as outlined in Knodler *et al.* (2014). In brief, paraffin embedded tissues were deparaffinized by heating to 60°C for 15 min, cleared with xylene, and rehydrated through an ethanol gradient to water. Antigen retrieval was performed in steam heated citrate buffer for 30mins, before cooling to room temperature and washing with water. Tissues were treated in PBS, 0.1% Triton X-100 and 0.05% Tween 20 for 15mins, then blocked with 5% donkey serum in PBS, 0.01% Triton X-100 and 0.05% Tween 20. Primary antibodies used were anti-Muc2 (1:200; Santa Cruz; H-300), anti-RegIIIβ (1:50; R&D Systems) and anti-Ecadherin (1:100, BD Biosciences) as well as the lectin UEA-1 (1:100; Vector Laboratories). Tissues were then probed with Alexa Fluor 488-conjugated donkey anti-goat IgG (1:1000; Life Technologies), Alexa Fluor 568-conjugated donkey anti-rabbit IgG (1:2000; Life Technologies) and/or Alexa Fluor 568-conjugated donkey anti-sheep IgG (1:2000; Life Technologies). Tissues were mounted using ProLong Gold Antifade reagent (Life Technologies) containing DAPI for DNA staining. Sections were viewed on a Zeiss AxioImager microscope and images taken using an AxioCam HRm camera operating through AxioVision software. Mucus layer thicknesses of Muc2 immunostained MethaCarn fixed colonic sections were quantified using AxioVision length from five mice, average thickness calculated from four separate sites evenly spaced around each colonic cross section, using mice from at least two independent experiments.

4.4.5 RNA extractions and quantitative real-time PCR

Immediately following euthanization of mice, cecal tissues were collected and placed in RNAlater (Qiagen), incubated at 4°C overnight, then stored at -80°C. Total RNA was extracted utilizing a RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Total RNA was quantified utilizing a NanoDrop microvolume spectrophotometer, and corresponding cDNA was synthesized using 0.5µg of RNA with 5× All-In-One RT MasterMix (Abm). For the qPCR reaction, 5µl of a 1:5 dilution of cDNA was added to 10 µl Bio-Rad SYBR green supermix with primers (final concentration, 300 nM; final volume, 20 µl), and qPCR was carried out using a Bio-Rad MJ MiniOpticon machine. Primers used were as follows: *Reg3β* (F - TGC CTA TGG CTC CTA TTG CT; R - CAC TCC CAT CCA CCT CTG TT), *Reg3γ* (F - TCC CAG GCT TAT GGC TCC TA; R - GCA GGC CAG TTC TGC ATC A) and *ll22* (F - ACC TTT CCT GAC CAA ACT CA; R - AGC TTC TCG CTC AGA CG). CFX Maestro software ver, 1.1 (Bio-Rad) was used for data quantification.

4.4.6 Enzyme-linked immunosorbent assay (ELISA)

Mice were infected as described above, 0.5-1 cm of the cecum excised, washed extensively in PBS then stored on ice in *ex vivo* secretion medium (FBS (10%, Sigma-Aldrich), RPMI (Gibco), Pen Strep (100 U/ml, Gibco), Sodium Pyruvate (1mM, Gibco), MEM non-essential amino acids (1X, Sigma-Aldrich), gentamicin (100µg/mL, Gibco)). Ceca and secretion medium were transferred under sterile conditions to a 24-well plate for 24h incubation at 37°C with 5% CO₂. Media was then collected and centrifuged at 4°C, supernatant collected and stored at -80°C. Protein concentration was estimated as described above and 65 µg of total protein probed per a well in duplicate performed according to the manufacturer's instructions (murine IL-22 ELISA MAXTM Deluxe Set; BioLegend).

4.4.7 Statistical analysis

All results presented in this study are expressed as the mean values ± standard errors (SEM) or standard deviations (SD) of the means where appropriate. Mann-Whitney U-test (to evaluate nonparametric data) and student t-test (to evaluate parametric data) were performed using GraphPad Prism software, version 7.02 for Windows. A p-value of 0.05 or less was considered significant, with asterisks denoting significance in figures.

Chapter 5: Conclusion

5.1 Summary

The intestinal epithelium consists of a single layer of cells that form an interface between the gut lumen and the underlying mucosal immune cells. It is often considered a passive barrier, simply providing a structural 'wall' to prevent unnecessary stimulation of underlying mucosal immune cells by antigenic products transiting through the intestinal lumen. However, the work outlined in this thesis demonstrates that IECs play an active and central role in the coordination of intestinal homeostasis through the use of the caspase-1 canonical and caspase-11(/-4) noncanonical inflammasomes to respond appropriately to enteric infection (Figure 5.1).



Figure 5.1 Intrinsic epithelial inflammasome activation coordinates mucosal immune defense.

Salmonella colonizes the intestinal epithelium and invades IECs. The majority of these bacteria are contained within the Salmonella-containing vacuole (SCV), however a proportion escape and readily multiple in the cytoplasm. These cytoplasmic Salmonella activate the inflammatory caspases through either 1) Naip-Nlrc4-Casp1 canonical inflammasome flagellar detection or 2) noncanonical Casp11 LPS detection. Regardless of inflammatory caspase activated, this cumulates in the processing and secretion of IL-18 as well as the processing of Gsdmd that self-assembles into small pores on the plasma membrane resulting in loss of membrane integrity. These disrupted IECs are actively extruded from the epithelium and are characterized by small condensed nuclei and 'ruffled' cellular borders. Simultaneously, the secreted IL-18 signals to underlying resident and recruited lymphoid cells to secrete IL-22. This IL-22 is detected by 1) goblet cells that bulk exocytose mucin as well as 2) enterocytes that upregulate and secrete C-type antimicrobial lectins (Reg3 β and γ). This coordinate response results in limiting Salmonella infection through mucin secretion physically 'pushing' infected shed IECs into the intestinal lumen away from the epithelium as well as exposing them to antimicrobial lectins.

Inflammasomes have been extensively studied in macrophages, but their role in other cell types have been poorly defined. Both human and murine intestinal epithelial cells express the noncanonical inflammasome (caspase-11 in mice, caspase-4 and -5 in humans), therefore Chapter 2 explored if these epithelial cells could employ their noncanonical inflammasome as an innate defense mechanism to guard against enteric infection. First, in a murine model of infectious colitis, higher Salmonella burdens were observed at intestinal sites in Casp11^{-/-} mice as compared to wildtype mice. Interestingly, cecal IEC colonization was dramatically different between wildtype and $Casp11^{-/-}$ mice. Wildtype crypts remaining relatively sterile with only occasional (and individual) intracellular bacteria present in apical IECs, whereas Casp11^{-/-} crypts contained numerous clusters of greater than five intracellular Salmonella. This phenotype appeared to be intrinsic to the epithelial cells themselves since siRNA-mediated depletion of caspase-4 (human orthologue) in a polarized intestinal epithelial monolayer also led to increased bacterial colonization. This increase in intracellular Salmonella levels correlated with decreased IEC shedding, suggesting IECs were utilizing caspase-4 to induce a form of pyroptosis (inflammatory cell death) to extrude compromised/infected cells to maintain the overall sterility of the monolayer. This phenomenon was also observed in vivo in a systemic Salmonella infection model, where the gallbladders of wildtype mice demonstrated extensive shedding of infected epithelial cells while the epithelium of $Casp11^{-/-}$ gallbladders remained relatively intact, despite carrying heavy loads of intracellular Salmonella. In addition to pyroptosis, activation of the noncanonical inflammasome led to processing and secretion of the proinflammatory cytokine IL-18, but not IL-1 β , by both human and murine IECs. Therefore, the IEC-intrinsic noncanonical inflammasome plays a critical role in antimicrobial defense at the intestinal

mucosal surface through pyroptotic shedding of infected IECs as well as secretion of IL-18 to prime and recruit professional immune cells to the site of infection.

In addition to caspase-11, murine IECs also express canonical inflammasome components. In a report published in the same issue of Cell Host and Microbe as the work outlined in Chapter 2, Dr. Wolf-Dietrich Hardt's group outlined a separate mechanism whereby IEC intracellular Salmonella were recognized by the Naip-Nlrc4 canonical inflammasome, ultimately activating caspase-1 that also resulted in pyroptosis of infected IECs (Sellin et al., 2014). Therefore, in Chapter 3 the interplay between IEC intrinsic canonical and noncanonical inflammasome signaling was explored. In an oral Salmonella infection model, $Casp 1^{-/-}$ and $Casp 1/11^{-/-}$ mice proved highly susceptible to infection at 18h and 72h p.i. $Casp11^{-/-}$ mice displayed high cecal pathogen burdens at 72h p.i., however, at 18h p.i. their cecal levels were not as high as those demonstrated by $Casp 1^{-/-}$ and $Casp 1/11^{-/-}$ mice This difference in burdens correlated with Casp1 and Casp11 transcript levels in which Casp11 transcripts were low at baseline but increased over the course of infection whereas Casp1 transcript levels were high at baseline and decreased after infection. Similar to the intestinal colonization phenotypes observed in Chapter 2, $Casp1/11^{-/-}$, $Casp1^{-/-}$ and $Casp11^{-/-}$ mice all carried higher IEC intracellular Salmonella loads and demonstrated lower levels of IEC shedding. To determine if this reflected IEC-intrinsic inflammasomes, enteroid monolayers were derived and infected with Salmonella. Casp11^{-/-} and wildtype monolayers responded similarly, whereas $Casp 1^{-/-}$ and $Casp 1/11^{-/-}$ monolayers carried significantly increased intracellular burdens, concomitant with marked decreases in IEC shedding and death. Pretreatment with IFN-y to mimic inflammation increased caspase-11 protein levels, leading to increased IEC shedding and reduced Salmonella burdens in Casp1^{-/-}

monolayers, while high intracellular burdens and limited cell shedding persisted in $Casp1/11^{-/-}$ monolayers. Thus, IEC employ both canonical and noncanonical inflammasomes for protection against enteric pathogens through IEC pyroptotic shedding. During a *Salmonella* infection, caspase-1 regulates early IEC inflammasome-mediated defense, while caspase-11 plays a role later in the course of infection once the pro-inflammatory response upregulates its expression.

Finally, Chapter 4 explored the overall impact of inflammasome activation on the intestinal mucosa. In an acute 18h oral infection model of *Salmonella*, wildtype mice demonstrate severe epithelial damage as well as a massive expulsion of mucins into the intestinal lumen, concurrent with a phenotypic loss of goblet cells. In contrast, *Casp1/11^{-/-}* mice displayed significantly higher intestinal pathogen burdens, concurrent with reduced epithelial damage, goblet cell loss and thinner mucus layers. Additional analysis revealed reduced production of the antimicrobial lectins *Reg3* β and *Reg3* γ as compared to wildtype mice, as well as reduced *Il22* transcript and protein levels. Wildtype mice treated with an IL-22 neutralizing antibody displayed reduced mucin and antimicrobial lectin responses similar to the levels observed in the inflammatory caspase-deficient mice. Thus, inflammasome signaling coordinates a multilayered innate defense at the intestinal mucosal surface, ultimately promoting IL-22 dependent signaling that protects the host during the early stages of an acute *Salmonella* infection.

This thesis details the mechanisms by which IEC intrinsic inflammasomes coordinates mucosal immune defense to protect against *Salmonella* (Figure 5.1). In context of the published literature, I envision the intestinal mucosal inflammasome specific response to proceed as follows: When *Salmonella* breach the cytoplasm of an IEC, this triggers the initial activation of the canonical

inflammasome through the intracellular Naip/Nlrc4/caspase-1 axis (Sellin et al., 2014, Crowley et al., 2016, Chapter 3). Over the course of infection, IECs throughout the infected tissues are primed for potential cytoplasmic insult through proinflammatory cytokine signalling (e.g. IL-18, IL-1 β , IFN γ) as well as the direct detection of basolateral pathogens by IECs through TLR recognition. These signals induce caspase-11 expression which binds intracellular Salmonella LPS, thereby activating the noncanonical inflammasome (Knodler et al., 2014, Shi et al., 2014, Chapter 2&3). Activation of both inflammatory caspases leads to the processing and secretion of the proinflammatory cytokine IL-18 as well as the induction of a specialized form of inflammatory cell death, termed pyroptosis (when seen in macrophages), in which infected IECs are shed to preserve the overall sterility of the epithelium (Sellin et al., 2014, Chapter 2&3). This mechanism potentially involves Gsdmd which is processed by the inflammasome and selfassembles into small pores, resulting in the release of the cytoplasmic contents, as well as PGE2 secretion, while trapping pathogens within the IEC cytoskeletal remnants (Rauch et al., 2017, Kayagaki et al., 2015, Shi et al., 2015, Jorgensen et al., 2016). Gsdmd pores also facilitate the secretion of processed IL-18 which recruits professional immune cells to the site of infection (such as neutrophils, natural killer (NK) and NK T-cells). It also signals to underlying innate lymphoid cells (ILC) to produce IFNy and TNFa (ILC1) or IL-22 (ILC3) and directly signals the nearby epithelium to induce goblet cell secretion of mucins (Muller et al., 2016, Kupz et al., 2014, Klose et al., 2013, Victor et al., 2017, Nowarski et al., 2015). At 18h p.i. IL-22 levels have significantly increased in the cecum, where they act on IECs to increase antimicrobial C-type lectin production (Reg3 β and γ) as well as induce the mass exocytosis of mucin granules from goblet cells (Chapter 4). This increased mucus secretion physically sweeps the shed IEC carcasses containing trapped intracellular Salmonella away from the epithelium as well as

exposing *Salmonella* to increased antimicrobial pressure from the C-type lectins, overall restricting *Salmonella* from overrunning the intestinal epithelium.

5.2 Significance

The worldwide burden of infectious foodborne disease is substantial, with non-typhoid *Salmonella* causing approximately 93.8 million cases of gastroenteritis annually that lead to approximately 155,000 deaths per year (Havelaar *et al.*, 2015, Kirk *et al.*, 2015, Majowicz *et al.*, 2010). In Canada, non-typhoid *Salmonella* is the third highest cause of foodborne illness and death and second highest contributor to foodborne infection-based hospitalizations (Thomas *et al.*, 2015). Here I described the characterization of the IEC intrinsic inflammasome and its fundamental role in the initiation and coordination of the host's innate intestinal defense response against *Salmonella*. Understanding this previously unappreciated component of the innate mucosal defense system expands our knowledge of the epithelium beyond that of a static physical barrier into a dynamically responding immune cell that protects the host from enteric disease through its own inherent mechanisms as well as its ability to extrinsically coordinate and recruit professional immune cells.

It is important to note, that although I have characterized the IEC intrinsic inflammasome and resulting mucosal response largely in the context of a *Salmonella* infection, this innate defense response will likely be launched against all enteric pathogens. I view the intestinal inflammasome as the first line of defense in the gut, halting the majority of infections once they reach this active barrier. However, in the case of severe infections where the IEC layer, and their intrinsic inflammasome based defenses are overrun, the epithelium can still serve as a stopgap
temporarily holding back the infection while simultaneously signaling and mobilizing professional immune cells to the compromised tissue site. Further study and characterization of the molecular mechanism underpinning this innate defense response is sorely needed and could potentially lead to novel therapeutics aimed at preventing foodborne illness.

5.3 Future directions

This thesis details the discovery of the IEC intrinsic inflammasome and its role in epithelial and mucosal host defense against *Salmonella* infection. However, various outstanding questions have been raised by this research and further work is required to address them.

One of the pressing issues from Chapter 2 and 3 concerns the differences between mouse and human IEC inflammasomes. In terms of the noncanonical inflammasome, there are key expression level differences between the two species. In mice, caspase-11 is not expressed at baseline and must be induced through nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), interferon regulatory factor 3 (IRF3), or interferon (type 1 and 2) signaling. In humans, there has been a gene duplication event producing two homologues of caspase-11; caspase-4 and -5 (Crowley *et al.*, 2017). Caspase-5 requires similar inflammatory signaling cues as caspase-11 while in contrast, caspase-4 is constitutively expressed (Crowley *et al.*, 2017). This indicates that our studies in Chapter 3 should be extended to include human organoids as a means to uncovering the contribution and interplay of canonical and noncanonical inflammasomes to human IEC host defense. In this context, the murine inflammasome mechanism described in Chapter 3 may not be truly reflective of the human IEC response and caspase-4 likely plays a larger role in human IEC intrinsic inflammasome restriction of *Salmonella* as suggested by studies using immortalized epithelial cells as noted in Chapter 2.

Another interesting route of investigation to arise from Chapter 3 regards IEC activation and its potential similarities to macrophage polarization. It is well established that IECs can differentiate into a variety of epithelial subsets of either absorptive or varying secretory cell lineages (Allaire et al., 2018). However, recent single cell transcriptomic papers are revealing a striking diversity among these previously assumed homogenous IEC subsets, suggesting these IEC play an as yet overlooked role in dynamically maintaining gut homeostasis (Parikh et al., 2019, Smillie et al., 2019). Alongside the work presented here, these findings suggest that IECs are an active immune cell and drive the initial innate immune response at the intestinal mucosa. Classical immunology divides macrophage activation into two broad cellular subsets: classically activated (M1) or alternatively activated (M2) (Martinez and Gordon, 2014). These roles were assigned to mimic Th cell nomenclature where Th1-derived IFNy signaling promoted cellular defense against intracellular infections while Th2-driven IL-4 mediated protection against extracellular parasitic infection. When stimulated with IFNy, similar to classical M1 macrophages, IECs increased their caspase-11 protein levels and therefore increased their ability to detect and respond to intracellular Salmonella (Chapter 3). "Alternatively activated" IECs have also been observed, by Schubart and colleagues, where they generated mice with IEC specific expression of a constitutively active version of STAT6 that dramatically increased worm clearance in vivo even in the absence of T-cells (Schubart et al., 2019). Further work is required to understand how closely IECs mimic classical immune cells to delineate their true contribution to innate defense

and for us to uncover manipulation and intervention strategies to better aid in the treatment of intestinal diseases.

Finally, deciphering the molecular underpinnings of IEC inflammasome-mediated cell shedding presents another exciting avenue of inquiry. Currently there have only been a handful of studies examining the proteolytic targets of the inflammatory caspase and the majority of these have focused on macrophages (Agard et al., 2010, Lorey et al., 2017, Burckstummer et al., 2009, Jamilloux et al., 2018). IECs present an interesting target for the study of their version of "pyroptotic" cell death due to cellular differences between epithelial cells and myeloid cells. There is fundamental overall differential protein expression between these cell types that change the identity, posttranslational modifications as well as concentration of the proteins the inflammatory caspases are exposed to. This further manifests in the cellular structural differences between professional inflammatory cells and IECs. Individual IECs form the intestinal epithelial barrier, therefore they are tightly linked through junction proteins as well as are polarized cells, presenting their apical face to the intestinal lumen and basolateral surface to underlying immune cells. This represents a novel situation for pyroptosis, where in macrophages, which are nonpolarized cells, it proceeds through caspase-1/11 activation followed by GsdmD cleavage and then localization of GsdmD N-terminal protein to the plasma membrane (Man and Kanneganti, 2015, He et al., 2015, Kayagaki et al., 2015, Shi et al., 2015). Here GsdmD associates with other cleaved N-termini to form a functional pore that compromises the integrity of the cellular membrane resulting in cell death. Based on the cellular morphology of IEC pyroptosis presented in Chapter 3, it does share some characteristics with macrophage pyroptosis (e.g. nuclei rounding and condensation) however it eventual cell death may be different in that its expulsion from the

epithelium appears controlled and phenotypically similar to IEC anoikis (de Vasconcelos *et al.*, 2019, Bertrand, 2011, Chapter 3). This suggests that the process of IEC pyroptosis is fundamentally different and characterizing how IEC polarization or inflammatory caspase interaction with cytoskeleton proteins could change how IEC pyroptosis occurs. In addition, further work uncovering novel IEC-specific proteolytic targets, the role of GsdmD cleavage in eventual cell death as well as potential interaction of the inflammatory caspases with tight junction proteins will be required to understand the mechanism of IEC pyroptosis.

5.4 Final remarks

Taken together, this thesis outlines the mechanisms by which *Salmonella* pathogenesis is restricted through epithelial intrinsic inflammasomes (Figure 5.1). When *Salmonella* breaches the cytoplasm of an IEC, it triggers both the canonical and noncanonical inflammasomes, ultimately leading to the processing and activation of caspase-1 and -11. This results in the apical controlled shedding of the infected cell as well as IL-18 secretion. The IL-18 release signals to underlying mucosal immune cells to produce IL-22 which feeds back to the epithelium prompting mucus secretion and antimicrobial lectin release. This innate defense mechanism promotes intestinal mucosal sterility through the expulsion of compromised IECs from the epithelium and physically restricts infected IECs in the lumen by increased mucus secretion as well as biochemically through antimicrobial production. Overall the intestinal epithelium utilizes inflammasome signaling to coordinate multiple layers of innate defense at the gut mucosal surface to ultimately restrict enteric pathogen infection and systemic spread.

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