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Short Article

Noncanonical Inflammasome Activation of Caspase-4/Caspase-11 Mediates Epithelial Defenses against Enteric Bacterial Pathogens

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SUMMARY

Inflammasome-mediated host defenses have been extensively studied in innate immune cells. Whether inflammasomes function for innate defense in intestinal epithelial cells, which represent the first line of defense against enteric pathogens, remains unknown. We observed enhanced *Salmonella enterica* serovar Typhimurium colonization in the intestinal epithelium of caspase-11-deficient mice, but not at systemic sites. In polarized epithelial monolayers, siRNA-mediated depletion of caspase-4, a human ortholog of caspase-11, also led to increased bacterial colonization. Decreased rates of pyroptotic cell death, a host defense mechanism that extrudes *S.* Typhimurium-infected cells from the polarized epithelium, accounted for increased pathogen burdens. The caspase-4 inflammasome also governs activation of the proinflammatory cytokine, interleukin (IL)-18, in response to intracellular (*S.* Typhimurium) and extracellular (enteropathogenic *Escherichia coli*) enteric pathogens, via intracellular LPS sensing. Therefore, an epithelial cell-intrinsic noncanonical inflammasome plays a critical role in antimicrobial defense at the intestinal mucosal surface.

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) (Hagar et al., 2013; Kayagaki et al., 2013); the cytosolic sensor for LPS is unknown. 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 Gram-negative bacterium *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) infects the IECs of several mammalian species, including humans (Santos et al., 2001), causing gastroenteritis. 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.

RESULTS

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 (Knodler et al., 2010) and IL-8 (Jung et al., 1995), assayed by ELISA. 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 *Salmonella*-induced IL-18 secretion (Figure S1A

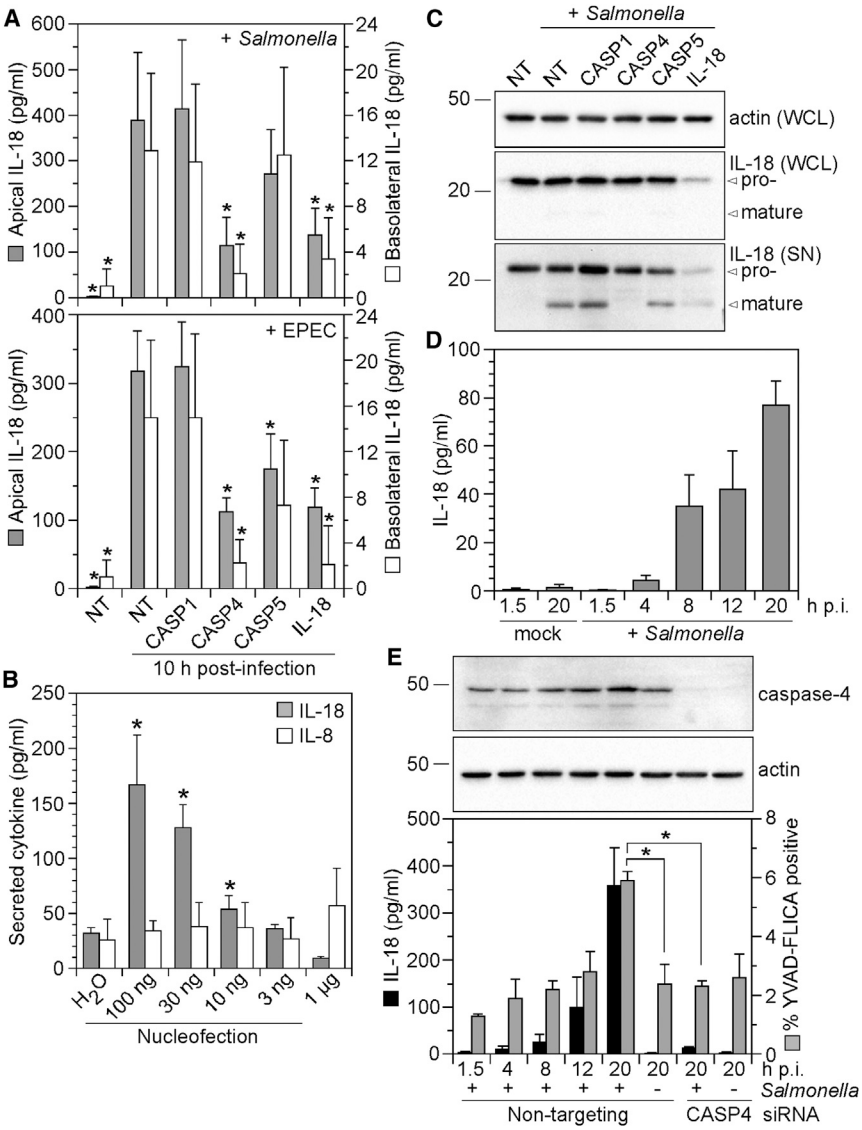

Figure 1. Caspase-4 Is Required for IL-18 Secretion and Processing in Human Intestinal Epithelial Cells

(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 hr pi 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 hr 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. HeLa cells were transfected with the indicated siRNA and infected 48 hr later with *S.* Typhimurium. Samples were collected at 10 hr 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 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 S1–S3.

available online), implicating inflammatory caspase catalytic activity. None of the inhibitors affected IL-8 secretion (Figure S1A).

mRNA and protein levels of inflammatory caspases in human epithelial cell lines and primary cells indicated caspase-1, -4, and -5 expression, with caspase-4 being the most abundant (Figure S2). Using small interfering RNA (siRNA) depletion of caspases (Figure S3) in polarized monolayers, knockdown of caspase-1 had no effect on *Salmonella*-induced IL-18 secretion, whereas caspase-4 knockdown significantly reduced it (Figure 1A). No difference in IL-8 secretion was observed (Figure S1B), demonstrating the selective impact of inflammatory caspase knockdown toward IL-18.

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 1C). siRNA-mediated knockdown of caspase-4, but not caspase-1 or -5, prevented the processing and release of mature IL-18. *Salmonella* infection also led to the time-dependent release of IL-18 from HCT 116 cells (Figure 1D), a colonic epithelial cell line that expresses only inflammatory caspase-4 (Figure S2), confirming that IL-18 secretion does not require caspase-1 in human IECs.

In mouse macrophages, cytosolic Gram-negative bacteria activate the caspase-11 inflammasome (Aachoui et al., 2013), via intracellular LPS detection (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 1B). Cytosolic LPS stimulated IL-18, but not IL-8, release in a dose-dependent manner (Figure 1B), 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 1A).

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 S2) and only modestly induced upon infection (Figure 1E),

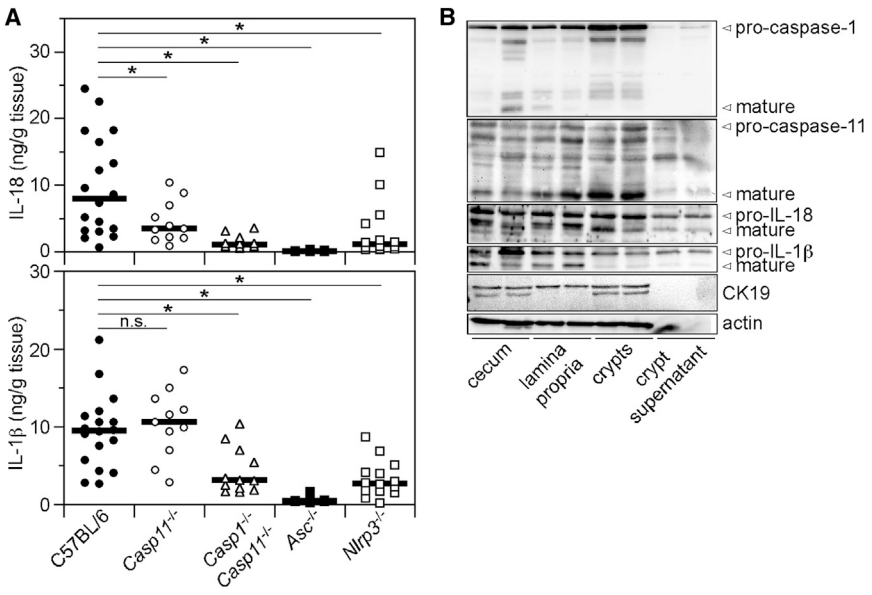

Figure 2. Caspase-11 Is Required for IL-18, but not for IL-1β, Secretion during Gut Inflammation

(A) Streptomycin-pretreated C57BL/6, *Casp11*$^{-/-}$, *Casp1*$^{-/-}$ *Casp11*$^{-/-}$, *Asc*$^{-/-}$, and *Nlrp3*$^{-/-}$ mice were orally infected with Δ*aroA S*. Typhimurium (3×10^6 cfu) and cecal tissues collected at 3 days pi. Tissues were washed and incubated in DMEM for 6 hr; 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 hr, and culture supernatants were collected. Protein extracts were analyzed by immunoblotting for pro- and mature forms of caspase-1, -11, IL-18, and IL-1β. Cytokeratin 19 (CK19) is a marker of epithelial cells, and actin is a loading control. Lysates from two representative mice are shown.

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 1E). Moreover, siRNA knockdown demonstrated that almost all secreted IL-18 and the majority of FAM-YVAD-FMK-positive cells at 20 hr postinfection (pi) was due to caspase-4 activity (Figure 1E). In support of recent findings (Kobayashi et al., 2013), we conclude that enteric bacterial infection of human IECs progressively activates caspase-4, resulting in IL-18 processing and secretion.

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 *Salmonella*-induced mouse model of gastroenteritis (Barthel et al., 2003; Miller et al., 1956-1957). Caspase-11 is the murine ortholog of human caspase-4 and -5 (Ng and Monack, 2013). IL-18 and IL-1β secretion from infected cecal tissues of wild-type C57BL/6, *Casp11*$^{-/-}$, or *Casp1*$^{-/-}$ *Casp11*$^{-/-}$ mice was quantified (Figure 2A). Secreted IL-18 levels were significantly lower in cecal explants from *Casp11*$^{-/-}$ mice compared to wild-type mice. IL-1β release was also detectable, consistent with the presence of various myeloid cells in cecal tissues, but there was no difference between wild-type and *Casp11*$^{-/-}$ mice (Figure 2A). By contrast, both IL-18 and IL-1β release from *Casp1*$^{-/-}$ *Casp11*$^{-/-}$ mouse cecal tissues were significantly reduced compared to wild-type 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-1β responses.

Pro-IL-1β 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-1β release were both significantly reduced from cecal explants compared to wild-type mice (Figure 2A), implicating ASC and NLRP3 in the release of these proinflammatory cytokines during *Salmonella*-induced gastroenteritis.

To determine the cellular origin of the secreted IL-1β and IL-18, whole crypts, enriched for IECs, were separated from the underlying lamina propria of cecal tissues from infected wild-type 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-1β, was also detected in crypt supernatants (Figure 2B). By contrast, expression of mature IL-1β and caspase-1 was comparatively increased in the lamina propria (Figure 2B). Therefore IL-18 activation and secretion primarily occurs in IECs in infected cecal tissues, correlating with mature caspase-11 localization. However, IL-1β production and activation during *Salmonella*-induced intestinal inflammation is predominantly associated with caspase-1 expression and processing in cells of the lamina propria.

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 hr pi, but caspase-1 and -5 had no effect (Figure 3B). Conversely, ectopic expression of caspase-4, but not caspase-1 or -5, restricted *Salmonella* growth (Figure 3A). Caspase-4 depletion did not affect recoverable bacteria ≤7 hr pi (Figure 3C), indicating no effect on bacterial internalization or early vacuolar trafficking events. Vacuolar replication of *Salmonella* was unperturbed by caspase-4 knockdown at 10 hr pi (Figure 3D, defined as <40 bacteria/cell; Malik-Kale et al., 2012), whereas cytosolic replication was enhanced (≥100 bacteria/cell; Knodler et al., 2014;

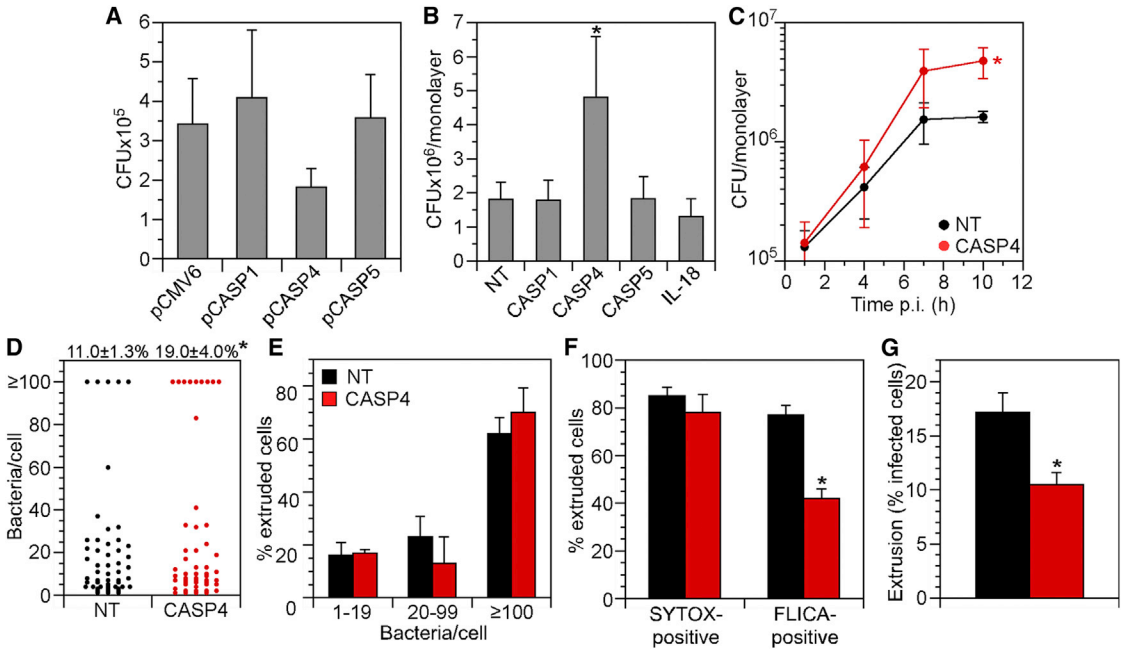

Figure 3. Caspase-4 Limits Bacterial Burdens via Epithelial Cell Shedding

(A) C2Bbe1 cells were nucleofected with pCMV6-XL5 (empty vector control), pCASP1, pCASP4, or pCASP5, infected with *S*. Typhimurium, and solubilized at 8 hr pi for enumeration of cfu. Mean ± SD.

(B and C) C2Bbe1 cells were treated with siRNA, polarized on semipermeable supports, and infected with *S*. Typhimurium. cfu were enumerated at 10 hr pi (B) or over a time course of infection (C). Mean ± SD. *$p < 0.01$ (B); $p < 0.02$ (C).

(D) C2Bbe1 cells were treated as in (B) and infected with mCherry *S*. Typhimurium. Monolayers were fixed at 10 hr 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 ± SD, n = 3 experiments). * $p < 0.05$.

(E and F) C2Bbe1 cells were treated as in (B) and infected with *S*. Typhimurium *glmS::mCherry* or *S*. Typhimurium *glmS::gfpmut3* for 10 hr. (E) Monolayers 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 ≥100 bacteria. Mean ± 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 ± SD *$p < 0.01$.

(G) C2BBe1 cells were treated and infected as in (D). At 9 hr p.i., monolayers were fixed and immunostained as in (D). The number of extruding/extruded infected cells was scored by fluorescence microscopy. Mean ± SD. *$p < 0.01$.

Malik-Kale et al., 2012) (Figure 3D). 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 1B and 3B), implicating pyroptosis instead. Most dying cells contained cytosolic *Salmonella* (≥100 bacteria/cell) (Figure 3E) and had a compromised plasma membrane (Figure 3F), 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 3F). Furthermore, the frequency at which infected cells were shed from epithelial monolayers was decreased in caspase-4-depleted cells (Figure 3G). 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.

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 wild-type and *Casp11*$^{-/-}$ mice (Figure 4A), 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 4A) and showed a significant reduction in histopathological features of cecal inflammation (Figure 4B). At early time points, initial colonization of cecal tissues by *S*. Typhimurium was dramatically different between wild-type and *Casp11*$^{-/-}$ mice (Figures 4C and 4D), specifically in epithelial cells (Figure 4E and 4F). 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 wild-type mice (1.4 epithelial cells/ten high-power fields, $p < 0.01$), suggesting that epithelial cell sloughing may be delayed in *Casp11*$^{-/-}$ mice.

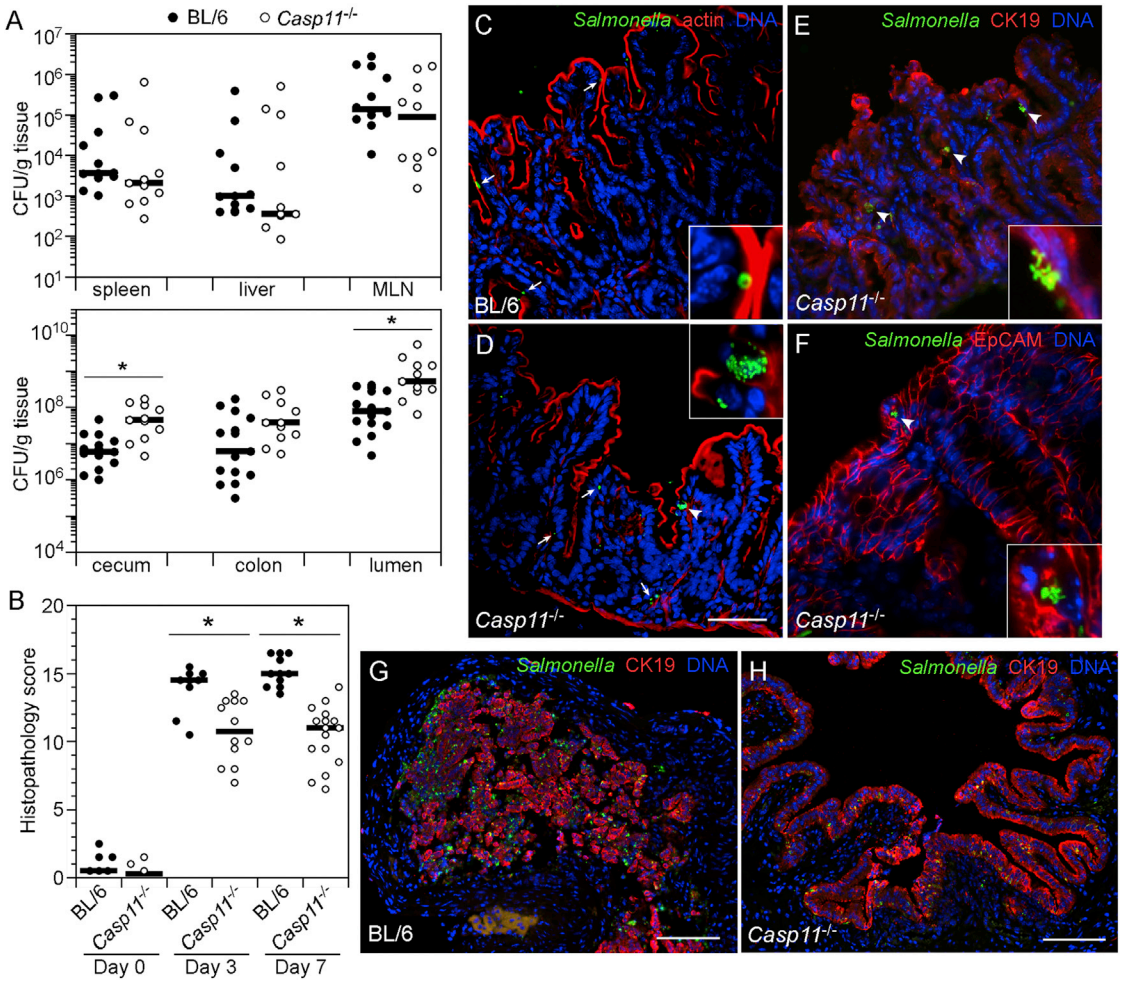

Figure 4. Caspase-11 Limits *S.* Typhimurium Burdens in the Gut

(A) Streptomycin-pretreated C57BL/6 and *Casp11*$^{-/-}$ mice were orally infected with Δ*aroA S.* Typhimurium (3 × 10^6 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 wild-type and *Casp11*$^{-/-}$ mice were infected as in (A). Semiquantitative 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 GFP-expressing wild-type *S.* Typhimurium (10^6 cfu) (green). Cecal tissues (day 1 pi.) 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 wild-type GFP-*Salmonella* (5 × 10^2 cfu). Gall bladders 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.

See also Figure S4.

The mouse gall bladder 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 wild-type mice, 7/29 *Casp11*$^{-/-}$ mice), wild-type and *Casp11*$^{-/-}$ mice showed histopathological differences (Figure S4). Gall bladders of wild-type mice showed extensive shedding of epithelial cells laden with bacteria (Figure 4G) and a heavy infiltration of neutrophils (Figure S4). Gall bladder epithelial cells of *Casp11*$^{-/-}$ mice were also filled with *Salmonella*, but relatively few had sloughed into the lumen (Figure 4H), and there was little evidence of neutrophil infiltration (Figure S4). Hence, caspase-11-induced epithelial shedding is important for the clearance of enteric bacteria at mucosal sites in vivo.

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 wild-type mice (Akhter et al., 2012) and succumb to *Burkholderia pseudomallei* and *Burkholderia thailandensis* infection, whereas wild-type mice do not (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 (Gu and Rosenblatt, 2012), and accelerated IEC turnover is a hallmark of infection with many enteric pathogens (Laughlin et al., 2014; Wallis et al., 1986; Ritchie et al., 2012; Kang et al., 2001). 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 (Kobayashi et al., 2013) have found that *S*. Typhimurium and EPEC both activate the caspase-4 inflammasome in human IECs. 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 (Lin et al., 2000; Yin et al., 2009), suggesting cell-type-specific or site-specific roles in inflammasome activation and inflammatory responses. Both caspase-4 and -5 are functional inflammasome components. Caspase-4 mediates inflammasome activation in keratinocytes (Sollberger et al., 2012); caspase-5 does so in THP-1 cells (Martinon et al., 2002); and both partially restrict *L. pneumophila* growth in THP-1 cells (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 S2), and only a minor increase in pro-caspase-4 is detected upon infection (Figure 1E). Although expression of caspase-11 is transcriptionally regulated by LPS in mouse macrophages and dendritic cells (Broz et al., 2012; Kayagaki et al., 2011), it is constitutively expressed at high levels in the mouse intestine (Kang et al., 2004). The relative abundance of caspase-4 and -11, and their constitutive expression in epithelial cells and the intestine (Figure S2) (Demon et al., 2014; Kang et al., 2004), might allow for the rapid sensing of enteric pathogens at mucosal sites.

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 (Bielaszewska et al., 2013), potentially explaining how the inflammasome senses such pathogens. 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 (Fassy et al., 1998), at the same processing site as human caspase-1 (Gu et al., 1997), which might account for our findings. 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.

EXPERIMENTAL PROCEDURES

Bacterial Strains

Wild-type and Δ*aroA Salmonella enterica* serovar Typhimurium SL1344 (Hoiseth and Stocker, 1981; Månsson et al., 2012), wild-type 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 wild-type 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 (Crépin 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).

Cell Culture

All cell lines were purchased from the American Type Culture Collection (ATCC). Caco-2 C2Bbe1 colorectal adenocarcinoma (ATCC CRL-2012), 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 μm 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 $\Omega.cm^2$ were used for infections.

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 hr 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 hr 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).

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 hr 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 hr prior to collection of cell-free supernatants.

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 μl) 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% CO_2 for 3 hr. Cells were infected with 1 μl EPEC subculture for 2.5 hr 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 μg/ml gentamicin.

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 wild-type 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 ~2.5 × 10^6 colony-forming units (cfu) of wild-type or Δ*aroA S.* Typhimurium SL1344 (Strep^R, in some cases carrying pFPV25.1), and sacrificed at specified times pi (Månsson et al., 2012). For gallbladder infections, mice were intravenously injected with ~500 cfu of wild-type *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 30 min 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.

Tissue Collection, Pathology Scoring, and Bacterial Counts

Tissue collection and bacterial counts were as described previously (Knodler et al., 2010; Månsson 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.

Statistical Analysis

The mean ± 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. A p value of less than 0.05 was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2014.07.002.

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