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HOST RESPONSE AND INFLAMMATION

Salmonella enterica Serovar Typhimurium Induces NAIP/NLRC4- and NLRP3/ASC-Independent, Caspase-4-Dependent Inflammasome Activation in Human Intestinal Epithelial Cells

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ABSTRACT *Salmonella enterica* serovar Typhimurium is a Gram-negative pathogen that causes diseases ranging from gastroenteritis to systemic infection and sepsis. *Salmonella* uses type III secretion systems (T3SS) to inject effectors into host cells. While these effectors are necessary for bacterial invasion and intracellular survival, intracellular delivery of T3SS products also enables detection of translocated *Salmonella* ligands by cytosolic immune sensors. Some of these sensors form multimeric complexes called inflammasomes, which activate caspases that lead to interleukin-1 (IL-1) family cytokine release and pyroptosis. In particular, the *Salmonella* T3SS needle, inner rod, and flagellin proteins activate the NAIP/NLRC4 inflammasome in murine intestinal epithelial cells (IECs), which leads to restriction of bacterial replication and extrusion of infected IECs into the intestinal lumen, thereby preventing systemic dissemination of *Salmonella*. While these processes are quite well studied in mice, the role of the NAIP/NLRC4 inflammasome in human IECs remains unknown. Unexpectedly, we found the NAIP/NLRC4 inflammasome is dispensable for early inflammasome responses to *Salmonella* in both human IEC lines and enteroids. Additionally, NLRP3 and the adaptor protein ASC are not required for inflammasome activation in Caco-2 cells. Instead, we observed a necessity for caspase-4 and gasdermin D pore-forming activity in mediating inflammasome responses to *Salmonella* in Caco-2 cells. These findings suggest that unlike murine IECs, human IECs do not rely on NAIP/NLRC4 or NLRP3/ASC inflammasomes and instead primarily use caspase-4 to mediate inflammasome responses to *Salmonella* pathogenicity island 1 (SPI-1)-expressing *Salmonella*.

KEYWORDS ASC, CASP4, NAIP, NLRC4, NLRP3, *Salmonella*, human innate immunity, inflammasome, intestinal epithelial cell

Enteric bacterial pathogens, such as *Salmonella enterica* serovar Typhimurium (here referred to as *Salmonella*), are leading causes of global morbidity and mortality from diarrheal diseases (1). Contracted upon ingestion of contaminated food or water, *Salmonella* colonizes the intestinal tract, where it uses evolutionarily conserved molecular syringes called type III secretion systems (T3SS) to inject effectors, or virulence factors, into the host cell cytosol (2). *Salmonella* contains two T3SS: the *Salmonella* pathogenicity island 1 (SPI-1) T3SS is expressed early in infection and enables *Salmonella* to invade host cells, while the SPI-2 T3SS is expressed at later time points during infection and allows *Salmonella* to replicate within host cells such as intestinal epithelial cells (IECs) (3–11). IECs thus serve as both the targets of and the first line of physical and innate immune defense against enteric pathogens like *Salmonella*. Most

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studies of *Salmonella*'s interactions with the innate immune system have been conducted in mice. However, key differences in innate immune genes carried by mice and humans make it unclear whether mice fully recapitulate how humans respond to *Salmonella*. Here, we interrogated how human IECs sense and respond to *Salmonella* infection.

The mammalian immune system can recognize invading intracellular pathogens through cytosolic sensors, such as the nucleotide-binding domain, leucine-rich repeat (NLR) receptors. Upon detecting a bacterial ligand or activity, these receptors oligomerize to form multimeric signaling complexes called inflammasomes (12). Inflammasomes recruit and activate cysteine proteases, such as caspase-1, caspase-4, and caspase-8 (12, 13). Certain inflammasomes require an adaptor protein called apoptosis-associated speck-like protein containing a CARD (ASC) to mediate their interaction with caspases (12). Active caspases can process some of the proinflammatory interleukin-1 (IL-1) family of cytokines, including IL-1β and IL-18 (12), and the pore-forming protein GSDMD (14–18). This leads to GSDMD-dependent release of IL-1 family cytokines and an inflammatory form of cell death known as pyroptosis (12). Release of these cytokines alerts nearby cells of the infection to promote inflammation, while pyroptosis can eliminate the pathogen's replicative niche within the infected host cell.

Various cellular insults during infection can trigger activation of different inflammasomes. Inflammasome activation is critical for control of *Salmonella* infection in mice (19). In both murine and human macrophages and murine IECs, *Salmonella* infection activates a family of inflammasome sensors termed NAIPs, whereby in mice, NAIP1 detects the *Salmonella* SPI-1 T3SS needle protein, NAIP2 detects the SPI-1 inner rod protein, and NAIP5 detects flagellin proteins (20–26). Activation of the NAIP/NLRC4 inflammasome specifically in murine IECs is both required and necessary to restrict *Salmonella* infection *in vivo*. NAIP/NLRC4 activation in IECs restricts intracellular bacterial replication, causes extrusion of infected cells from the intestinal epithelial layer, and prevents dissemination of *Salmonella* to systemic sites (27–29). In contrast to mice, humans encode a single NAIP inflammasome sensor, which promiscuously recognizes the T3SS needle, inner rod, and flagellin proteins in human macrophages (24, 30–34). Patients with activating mutations in *NLRC4* exhibit intestinal pathology, suggesting that the NAIP/NLRC4 inflammasome may play a role in human intestinal immunity (35–38). However, the role of the NAIP/NLRC4 inflammasome in human IECs during *Salmonella* infection remains unknown.

Salmonella infection of macrophages can also induce the NLRP3 inflammasome, which is activated by a variety of stimuli during infection, including potassium efflux (39–45). In murine macrophages, the NLRP3 inflammasome is thought to be important for late time points during *Salmonella* infection (44). The NLRP3 inflammasome can also be secondarily activated by activation of the noncanonical inflammasome (caspase-11 in mice or caspase-4/5 in humans) in response to cytosolic lipopolysaccharide (LPS) (46–52). In murine macrophages and murine and human IECs, the murine caspase-11 or human caspase-4/5 inflammasome is activated during *Salmonella* infection (53–56). In human macrophages, *Salmonella* infection triggers recruitment of both NLRC4 and NLRP3 to the same macromolecular complex (57). However, whether the NLRP3 inflammasome plays a functional role during *Salmonella* infection of human IECs has not been previously tested.

Human IECs infected with *Salmonella* undergo caspase-4 inflammasome activation at both early (1.5 h postinfection [hpi]) and late (10 hpi) time points following infection (53, 55). However, whether other inflammasomes, such as the NAIP/NLRC4 or NLRP3 inflammasomes, are involved in this response has not been previously investigated. In this study, we have found that although human IECs undergo inflammasome activation in response to SPI-1-expressing *Salmonella*, typical NAIP/NLRC4 activators such as T3SS ligands and flagellin were not sufficient to activate the inflammasome in the human intestinal Caco-2 cell line or human intestinal enteroids. Additionally, using a combination of pharmacological inhibitors and CRISPR/Cas9 technology, we found that the NAIP inflammasome, the NLRP3 inflammasome, and the adaptor protein ASC

are all dispensable for early inflammasome responses to SPI-1-expressing *Salmonella* in Caco-2 cells. Instead, we observed that caspase-1 partially contributed, whereas caspase-4 is absolutely necessary for inflammasome activation in Caco-2 cells in response to *Salmonella* infection. Our findings delineate the role of several inflammasomes in human IECs during *Salmonella* infection. Importantly, these findings indicate how widely inflammasome responses to infection can vary between species as well as cell types.

RESULTS

***Salmonella* infection induces SPI-1-dependent inflammasome activation in human intestinal epithelial cells.** Once inside the host, *Salmonella* upregulates expression of its SPI-1 T3SS, which delivers effectors that enable *Salmonella* to invade intestinal epithelial cells (3, 6, 7, 9, 58–60). In human macrophages, the SPI-1 T3SS is required for inflammasome activation during *Salmonella* infection (33, 34). Human IECs infected with *Salmonella* undergo inflammasome activation, but whether the SPI-1 T3SS is required for this response remains unknown (53, 55). To test if inflammasome activation in response to *Salmonella* infection of human IECs requires the SPI-1 T3SS, we infected wild-type (WT) Caco-2 cells, a human colorectal cell line, with WT *Salmonella* Typhimurium (WT Stm) or *Salmonella* lacking its SPI-1 T3SS translocation activity (Δ*sipB* Stm) and assayed for inflammasome activation by measuring release and cleavage of the inflammasome-dependent cytokine IL-18 at 6 hpi (Fig. 1A and B). Cells infected with WT Stm released significantly increased levels of cleaved IL-18 into the supernatant compared to mock-infected cells (Fig. 1A and B). In contrast, cells infected with Δ*sipB* Stm failed to release cleaved IL-18 (Fig. 1A and B). We also assayed for release of the inflammasome-dependent cytokine IL-1β but found that Caco-2 cells failed to release detectable IL-1β (see Fig. S1A in the supplemental material), perhaps due to low IL-1β expression in human IECs (53, 55). We measured cell death as another readout of inflammasome activation by assaying for uptake of the cell-impermeant dye propidium iodide (PI), which enters cells as they form pores in their plasma membrane and undergo cell death. In cells infected with WT Stm, PI uptake began to occur between 4 and 6 hpi and gradually increased over time, indicating that infected cells begin undergoing cell death as early as 4 to 6 hpi (Fig. 1C). In contrast, cells infected with Δ*sipB* Stm did not uptake any PI (Fig. 1C). Notably, bacterial uptake of Δ*sipB* Stm into Caco-2 cells was significantly lower than bacterial uptake of WT Stm (Fig. S1B), consistent with previous findings (53, 61, 62). Additionally, we found that inflammasome activation in response to *Salmonella* also occurred in a SPI-1-dependent manner in another human colorectal cell line, T84 cells (Fig. 1D). T84 cells infected with SPI-1-expressing WT Stm also released IL-18 into the supernatant at 6 hpi but failed to release any IL-18 in response to Δ*sipB* Stm (Fig. 1D). Similar to Caco-2 cells, uptake of Δ*sipB* Stm into T84 cells was significantly lower than that of WT Stm (Fig. S1C). As a control, we found that in both Caco-2 and T84 cells, the inflammasome-independent cytokine IL-8 is released in response to both WT and Δ*sipB* Stm (Fig. S1D and E).

Inflammasome activation leads to cleavage of the pore-forming protein GSDMD (16–18). The N-terminal fragment of cleaved GSDMD inserts into the host cell plasma membrane and oligomerizes to create a pore through which cellular components such as cleaved IL-1 and IL-18 can be released (63–66). These pores eventually cause rupture of the cell through osmotic flux, resulting in pyroptosis (12). To determine if pore formation by GSDMD is required for release of IL-18 and PI uptake in human IECs infected with *Salmonella*, we pretreated Caco-2 cells with the chemical inhibitor disulfiram, which prevents cleaved GSDMD from inserting into the plasma membrane and thus abrogates pore formation (67). Treatment with disulfiram led to loss of IL-18 release and PI uptake during *Salmonella* infection (Fig. 1E and F). Importantly, release of the inflammasome-independent cytokine IL-8 was not affected by disulfiram treatment (Fig. S1F). Collectively, these results suggest that human IECs undergo SPI-1-dependent inflammasome activation and that GSDMD-mediated pore formation is required for IL-18 release and PI uptake in response to *Salmonella* in human IECs.

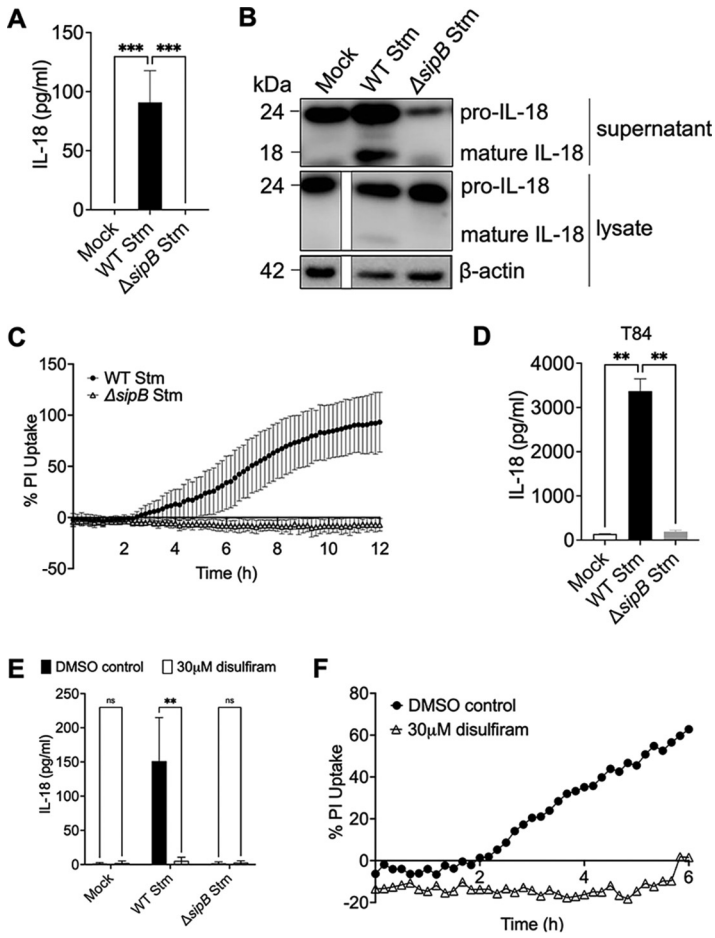

FIG 1 *Salmonella* infection induces SPI-1-dependent inflammasome activation in human intestinal epithelial cells. Caco-2 cells (A to C, E, and F) or T84 cells (D) were infected with PBS (Mock), WT *S.* Typhimurium, or Δ*sipB S.* Typhimurium. (A and D) Release of IL-18 into the supernatant was measured by ELISA at 6 hpi. (B) Lysates and supernatants collected 6 hpi were immunoblotted for IL-18 and β-actin. (C) Cell death was measured as percentage of cells that have uptaken propidium iodide (% PI uptake), normalized to cells treated with 1% Triton. (E and F) Caco-2 cells were treated with 30 μM disulfiram or DMSO as a vehicle control 1 h prior to infection. Cells were then infected with PBS (Mock), WT *S.* Typhimurium, or Δ*sipB S.* Typhimurium. (E) Release of IL-18 into the supernatant was measured by ELISA at 6 hpi. (F) Cell death was determined by % PI uptake, normalized to cells treated with 1% Triton. ns, not significant; **, $P < 0.01$; ***, $P < 0.001$, by Dunnett's multiple-comparison test (A and D) or by Šídák's multiple-comparison test (E). Shown are the averages and error bars representing the standard deviation for at least three pooled independent experiments.

Bacterial T3SS ligands do not activate the NAIP/NLRC4 inflammasome in human intestinal epithelial cells. We next sought to determine the bacterial ligands that trigger inflammasome activation in human IECs. The *Salmonella* SPI-1 T3SS inner rod protein (PrgJ), the SPI-1 T3SS needle protein (PrgI), and flagellin activate the NAIP/NLRC4 inflammasome in murine macrophages and IECs as well as human macrophages (25, 26, 28). Given that the SPI-1 T3SS was required for *Salmonella* to trigger inflammasome activation in human IECs (Fig. 1), we hypothesized that this response was due to NAIP-mediated recognition of *Salmonella* T3SS ligands. First, we asked whether *Salmonella* T3SS ligands are sufficient to activate the inflammasome in human IECs. We used the Gram-positive bacterium *Listeria monocytogenes* to deliver *Salmonella* T3SS ligands into human IECs. We have previously used the *Listeria* system to deliver *Salmonella* T3SS ligands into the cytosol of human macrophages in order to activate the human NAIP inflammasome (33, 34). In this system, ligands of interest are translationally fused to the N terminus of truncated ActA, enabling the ligands to be delivered into the host cell cytosol (33, 68). Human macrophages infected with *Listeria* expressing the *Salmonella* SPI-1 T3SS inner rod PrgJ or needle PrgI undergo robust inflammasome activation (33). Surprisingly, IECs infected with *Listeria*

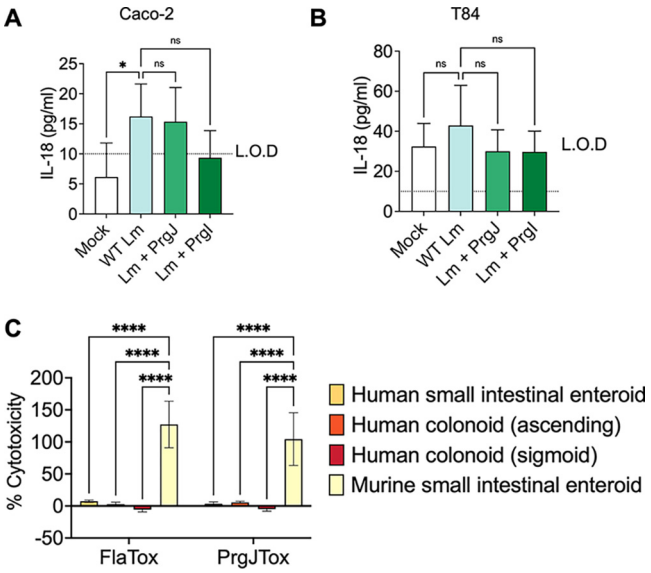

FIG 2 Bacterial T3SS ligands do not activate the inflammasome in human intestinal epithelial cells. (A and B) Caco-2 (A) or T84 (B) cells were primed for 3 h with 100 ng/mL of Pam3CSK4 and infected with PBS (Mock), WT *L. monocytogenes* (WT Lm), or *L. monocytogenes* expressing *S.* Typhimurium SPI-1 inner rod (Lm + PrgJ), or SPI-1 needle (Lm + PrgI), at an MOI of 100. Release of IL-18 into the supernatant was measured by ELISA at 16 hpi. L.O.D indicates the limit of detection of the assay. (C) Differentiated intestinal enteroids or colonoids were treated with FlaTox (PA + LFn-Fla) or inner rod Tox (PA + LFn-Rod) in medium containing propidium iodide for 4 h. Cell death was measured as percent uptake of propidium iodide, normalized to enteroids or colonoids treated with 1% Triton. ns, not significant; *, $P < 0.05$; ****, $P < 0.0001$ by Dunnett's multiple-comparison test. Shown are the averages and error bars representing the standard deviation from at least three pooled independent experiments.

expressing either the SPI-1 inner rod (PrgJ) or needle (PrgI) proteins failed to induce IL-18 release (Fig. 2A and B; see also Fig. S2A and B). As a positive control, we infected human monocyte-derived macrophages (hMDMs) with *Listeria* expressing the SPI-1 inner rod PrgJ and observed increased inflammasome activation relative to cells infected with WT *Listeria* (WT Lm) (Fig. S2C). Polarized Caco-2 cells and C2Bbe1 cells, a Caco-2 subtype, infected with *Listeria* expressing the SPI-1 inner rod PrgJ also failed to release IL-18 levels above those observed in cells infected with WT Lm (Fig. S2D and E). Collectively, these data indicate that *Listeria* delivery of bacterial T3SS ligands is not sufficient to induce inflammasome activation in human IEC lines.

As we did not observe inflammasome activation in Caco-2 cells by bacterial T3SS ligands delivered using *Listeria*, we tested a second method, the *Bacillus anthracis* toxin system, to deliver these bacterial ligands into the cytosol of IECs (69). The anthrax toxin delivery system contains two subunits: a protective antigen (PA) that creates a pore in the host endosomal membrane and a truncated lethal factor (LFn) that is delivered through the PA pore into the cytosol. T3SS ligands that are translationally fused to the N-terminal domain of the *B. anthracis* LFn are delivered into the host cell cytosol upon treatment with both PA and the LFn fusion (69) (collectively referred to as Tox). We delivered the *Salmonella* SPI-1 T3SS inner rod protein (PrgJTox) into Caco-2 cells (Fig. S3A), polarized Caco-2 cells (Fig. S3B), C2Bbe1 cells (Fig. S3C), and T84 cells (Fig. S3D). In all cell types, we failed to observe IL-18 secretion in response to PrgJTox that was above that of the PA-alone control (Fig. S3). Thus, our data indicate that human IEC lines do not undergo inflammasome activation in response to bacterial T3SS ligands delivered with the anthrax toxin system.

To determine if this absence of inflammasome responses to T3SS ligands was limited to immortalized IECs or extended to nonimmortalized IECs as well, we delivered flagellin (FlaTox) or the SPI-1 T3SS inner rod PrgJ (PrgJTox) into human intestinal enteroids and colonoids and measured levels of cell death as a readout for inflammasome

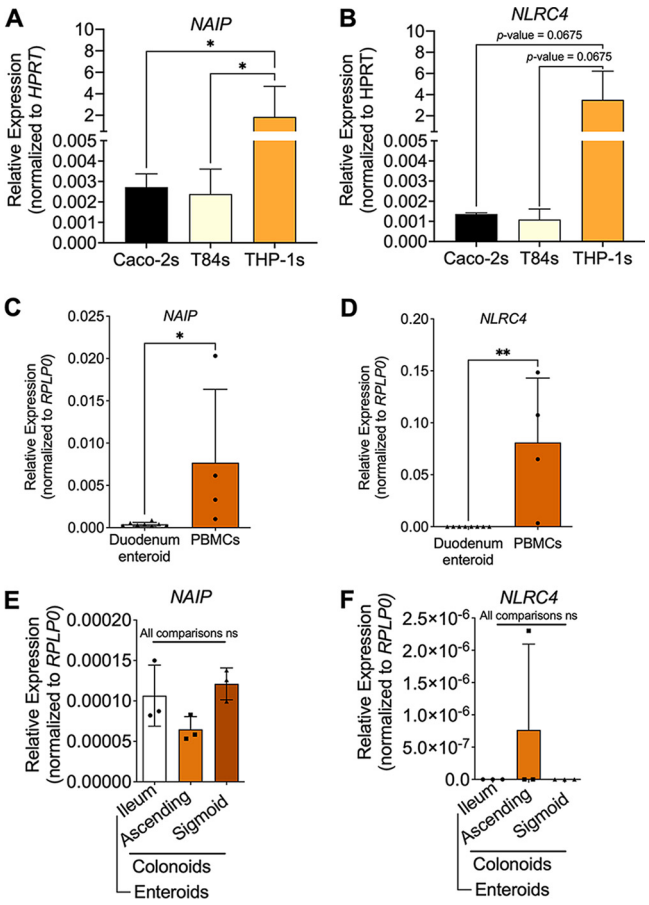

FIG 3 Human intestinal epithelial cells express low levels of NAIP/NLRC4 compared to human myeloid cells. mRNA expression of *NAIP* and *NLRC4* relative to the housekeeping control *HPRT* or *RPLP0* as measured by RT-qPCR in Caco-2 cells, T84 cells, and THP-1 macrophages (A and B); in human peripheral blood mononuclear cells (PBMCs) and human duodenal enteroids (C and D); and in ileal enteroids and colonoids (F and G). ns, not significant; *, $P < 0.05$; **, $P < 0.01$ by Kruskal-Wallis test (A), Dunnett's multiple-comparison test (B), unpaired *t* test (C and D), or Tukey's multiple-comparison test (E and F). Shown are the averages and error bars representing the standard deviation for at least three pooled independent experiments.

activation (Fig. 2C). Both human small intestinal enteroids and colonoids failed to undergo cell death when treated with FlaTox or PrgJTox (Fig. 2C). In contrast, murine intestinal enteroids underwent robust cell death in response to FlaTox or PrgJTox, as expected (28), indicating that these preparations of FlaTox and PrgJTox had the expected biological activity but were not able to activate the inflammasome in primary or transformed human IECs (Fig. 2C). Collectively, our data demonstrate that unlike murine IECs, human IECs fail to undergo NAIP/NLRC4 inflammasome activation in response to bacterial T3SS ligands.

Human intestinal epithelial cells express low levels of *NAIP* and *NLRC4* compared to human myeloid cells. Given the role of NAIP/NLRC4 in detecting and responding to *Salmonella* and bacterial T3SS ligands in murine IECs as well as murine and human macrophages (21–26, 28, 32–34), our findings that human IECs do not respond to T3SS ligands were surprising. Thus, we next asked if expression of *NAIP* and *NLRC4* in human IECs is comparable in Caco-2 cells, T84 cells, and human THP-1 macrophages (Fig. 3A and B). Consistent with their poor responsiveness to cytosolic delivery of NAIP ligands, both Caco-2 and T84 cells expressed very low levels of *NAIP* and *NLRC4* mRNA compared to human macrophages. Moreover, primary small intestinal enteroids and colonoids also expressed very low levels of *NAIP* and *NLRC4* compared to human peripheral blood mononuclear cells (PBMCs), indicating that this low *NAIP*

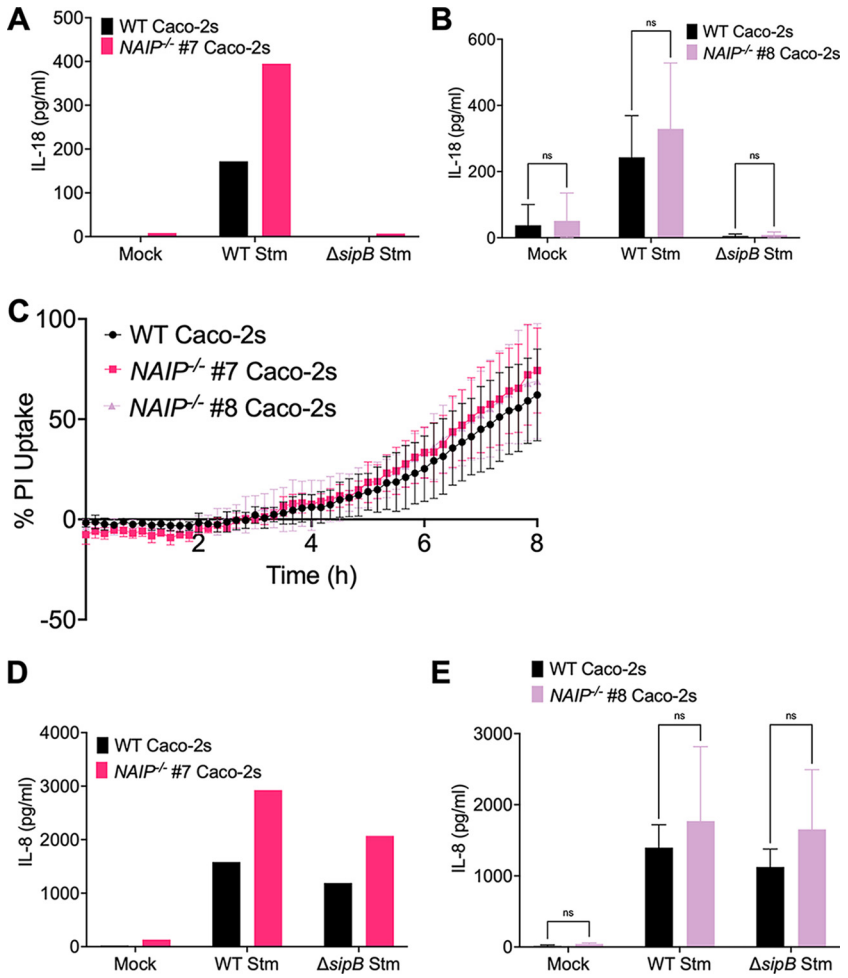

FIG 4 NAIP is not required for inflammasome responses to *Salmonella* in human intestinal epithelial cells. WT or two independent single-cell clones of *NAIP*$^{-/-}$ Caco-2 cells were infected with PBS (Mock), WT *S.* Typhimurium, or Δ*sipB S.* Typhimurium for 6 h. (A, B, D, and E) Release of IL-18 (A and B) or IL-8 (D and E) into the supernatant was measured by ELISA. (C) Cell death was measured as percent uptake of propidium iodide, normalized to cells treated with 1% Triton. ns, not significant by Šídák's multiple-comparison test. Shown are the averages and error bars representing the standard deviation for at least three pooled independent experiments.

and *NLRC4* expression was a general feature of human IECs (Fig. 3C to F). Collectively, these data suggest that human IECs express very low levels of NAIP and NLRC4, and this may partially explain why human IECs do not mount inflammasome responses to T3SS ligands.

NAIP is not required for inflammasome activation in response to *Salmonella* infection of human intestinal cells. Our data thus far indicate that human IECs do not undergo NAIP/NLRC4 inflammasome activation in response to individual T3SS ligands and express low levels of the *NAIP* and *NLRC4* genes. However, to formally test if the inflammasome activation we observe in human IECs infected with *Salmonella* (Fig. 1) requires NAIP, we used the Clustered Regularly Interspersed Palindromic Repeat (CRISPR) system, in conjunction with the RNA-guided exonuclease Cas9, to disrupt the *NAIP* gene in Caco-2 cells (Fig. S4). We sequenced two independent single-cell clones each of *NAIP*$^{-/-}$ Caco-2 cells (*NAIP*$^{-/-}$ #7, *NAIP*$^{-/-}$ #8) to confirm appropriate targeting of *NAIP* in each line (Fig. S4). Caco-2 cells are polyploid, and we therefore found multiple mutant alleles for each CRISPR clone we sequenced (Fig. S4). For the two *NAIP*$^{-/-}$ clones, all the changes resulted in a premature stop codon (Fig. S4).

We next infected WT or *NAIP*$^{-/-}$ Caco-2 cells with WT or Δ*sipB* Stm (Fig. 4). As

expected, WT Caco-2 cells infected with WT Stm released significantly increased levels of IL-18 and underwent cell death, as measured by PI uptake (Fig. 4A to C), and this response was dependent on the presence of the SPI-1 T3SS, as cells infected with Δ*sipB* Stm failed to undergo inflammasome activation (Fig. 4A and B). In *NAIP*$^{-/-}$ Caco-2 cells infected with WT Stm, we did not observe a decrease in inflammasome activation compared to WT Caco-2 cells (Fig. 4A to C). WT and *NAIP*$^{-/-}$ Caco-2 cells secreted similar levels of the inflammasome-independent cytokine IL-8 (Fig. 4D and E). Overall, these data indicate that NAIP is not required for inflammasome responses to SPI-1-expressing *Salmonella* in human IECs.

NLRP3 and ASC are dispensable for inflammasome activation in response to *Salmonella* in human intestinal epithelial cells. Since the NAIP/NLRC4 inflammasome did not have a role in responding to *Salmonella* grown under SPI-1-inducing conditions in human IECs, we sought to determine whether another host cytosolic sensor could be responsible for inflammasome activation. One candidate is the NLRP3 inflammasome, which can be activated by a variety of stimuli during infection, including potassium efflux (39–43). In both murine and human macrophages, both the NAIP/NLRC4 and NLRP3 inflammasomes are activated during *Salmonella* infection (34, 44, 45, 57, 70, 71). To determine if the inflammasome activation we observed in human IECs is dependent on the NLRP3 inflammasome, we infected WT Caco-2 cells that were pretreated with MCC950, a potent chemical inhibitor of the NLRP3 inflammasome (72), and measured IL-18 release (Fig. 5A). As expected, cells treated with the dimethyl sulfoxide (DMSO) control underwent robust inflammasome activation in response to WT Stm infection (Fig. 5A). Interestingly, cells treated with various concentrations of MCC950 exhibited similar levels of inflammasome activation as DMSO control-treated cells (Fig. 5A), suggesting that the NLRP3 inflammasome is not required for inflammasome responses to *Salmonella* in human IECs. To determine whether priming was required to observe a role for the NLRP3 inflammasome, we primed Caco-2 cells with the TLR1/2 agonist Pam3CSK4 prior to MCC950 treatment and *Salmonella* infection (Fig. S5A). Like in unprimed cells, primed cells infected with WT Stm underwent robust inflammasome activation under both DMSO-treated and MCC950-treated conditions, suggesting that priming with Pam3CSK4 does not affect inflammasome activation in response to *Salmonella* infection of Caco-2 cells. Additionally, we found that like *NAIP* and *NLRC4*, mRNA expression of *NLRP3* is also very low in Caco-2 cells compared to THP-1 macrophages (Fig. 5B), which may explain why NLRP3 does not contribute to inflammasome responses to *Salmonella* in human IECs.

In addition to the NAIP/NLRC4 and NLRP3 inflammasomes, there are many other inflammasomes that can get activated in response to bacterial infections. The majority of these inflammasomes, including AIM2, IFI16, NLRP3, NLRP6, NLRP7, and pyrin, use an adaptor protein called ASC to recruit and activate downstream caspases (13). To determine if ASC-dependent inflammasomes participate in the response to *Salmonella* in human IECs, we tested if ASC is required for inflammasome activation. Using CRISPR/Cas9, we disrupted *PYCARD*, the gene that encodes ASC, in Caco-2 cells (Fig. S6). We sequence validated two independent single-cell clones of *PYCARD*$^{-/-}$ Caco-2 cells (*PYCARD*$^{-/-}$ #4, *PYCARD*$^{-/-}$ #6) (Fig. S6). Most of the mutations resulted in a premature stop codon, or the mutated protein bore no resemblance to the WT protein sequence (Fig. S6). mRNA expression of *PYCARD* was also abrogated in the knockout (KO) clones relative to WT Caco-2 cells (Fig. S7A).

We next infected WT or *PYCARD*$^{-/-}$ Caco-2 cells with WT or Δ*sipB* Stm and assayed for inflammasome activation (Fig. 5C and D). As expected, WT Caco-2 cells infected with WT Stm released significantly increased levels of IL-18 and underwent cell death (Fig. 5C and D). This was dependent on the presence of the SPI-1 T3SS, as cells infected with Δ*sipB* Stm failed to undergo inflammasome activation (Fig. 5C and D). In *PYCARD*$^{-/-}$ Caco-2 cells infected with WT Stm, we did not observe a decrease in inflammasome activation compared to WT Caco-2 cells, indicating that ASC is dispensable for IL-18 release and cell death in Caco-2 cells in response to *Salmonella* Typhimurium. WT and *PYCARD*$^{-/-}$ Caco-2 cells released similar levels of the inflammasome-independent cytokine IL-8 (Fig. 5E). In addition,

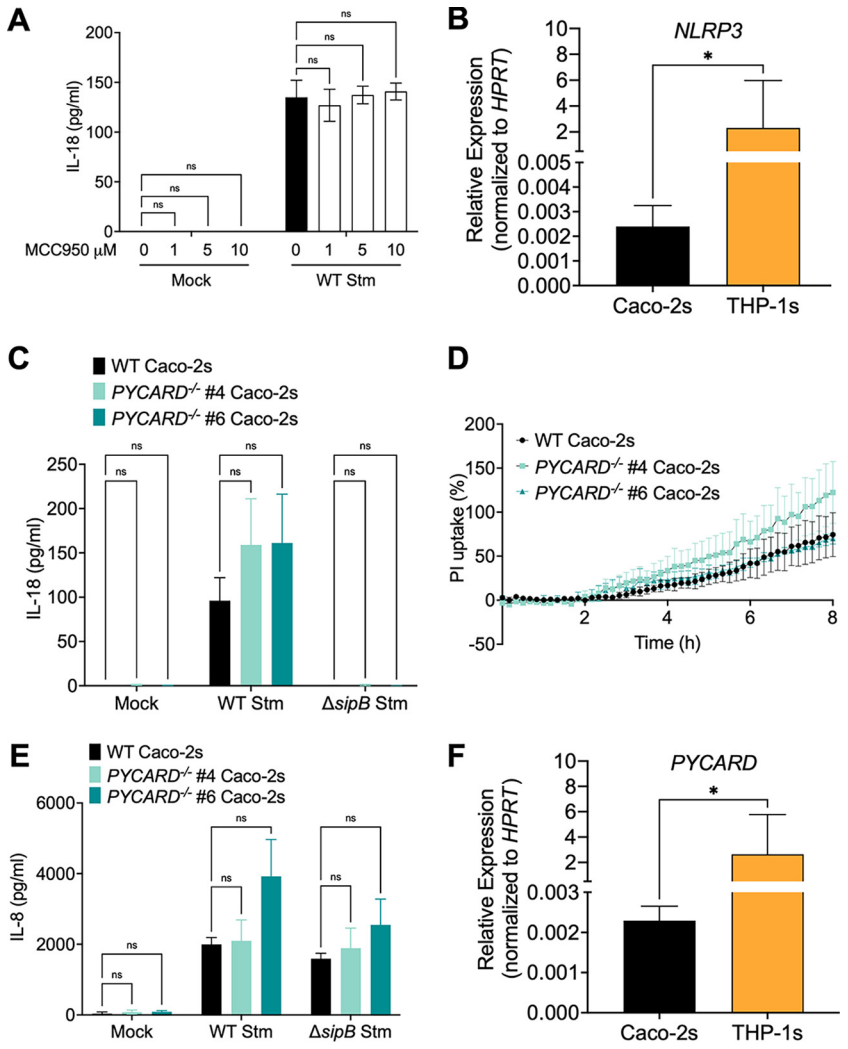

FIG 5 NLRP3 and ASC are dispensable for inflammasome responses to *Salmonella* in human intestinal epithelial cells. (A) One hour prior to infection, WT Caco-2 cells were treated with the indicated concentrations of MCC950 or DMSO as a vehicle control. Cells were then infected with PBS (Mock) or WT *S.* Typhimurium for 6 h. Release of IL-18 into the supernatant was measured by ELISA. (B and F) mRNA expression of *NLRP3* and *PYCARD* relative to the housekeeping control *HPRT* as measured by RT-qPCR in Caco-2 cells and THP-1 macrophages. (C to E) WT or two independent single-cell clones of *PYCARD*$^{-/-}$ Caco-2 cells were infected with PBS (Mock), WT *S.* Typhimurium, or Δ*sipB S.* Typhimurium. (C and E) Release of IL-18 or IL-8 into the supernatant was measured by ELISA at 6 hpi. (D) Cell death was measured as percent uptake of propidium iodide, normalized to cells treated with 1% Triton. ns, not significant; *, $P < 0.05$ by Dunnett's multiple-comparison test (A, C, and E), or Mann-Whitney test (B and F). Shown are the averages and error bars representing the standard deviation for at least three pooled independent experiments.

expression of *PYCARD* mRNA in Caco-2 cells was very low compared to that in THP-1 macrophages (Fig. 5F), and we did not detect ASC protein expression even in WT Caco-2 cells, indicating that ASC is expressed at very low levels in human IECs (Fig. S7B). Collectively, these data indicate that NLRP3- and ASC-dependent inflammasomes are not required for inflammasome responses to *Salmonella* infection in human IECs.

Caspase-1 is not a major contributor to inflammasome activation in response to *Salmonella* in human intestinal epithelial cells. Inflammasomes can recruit various caspases, which can then process IL-1 family cytokines and gasdermin-D and mediate pyroptosis (14–18, 73–75). For example, the murine NAIP/NLRC4 inflammasome can recruit both caspase-1 and caspase-8 in response to *Salmonella* infection of murine IECs (28). In human macrophages, NAIP/NLRC4, NLRP3, caspase-1, and caspase-8 are recruited to the same macromolecular complex during *Salmonella* infection (57). We

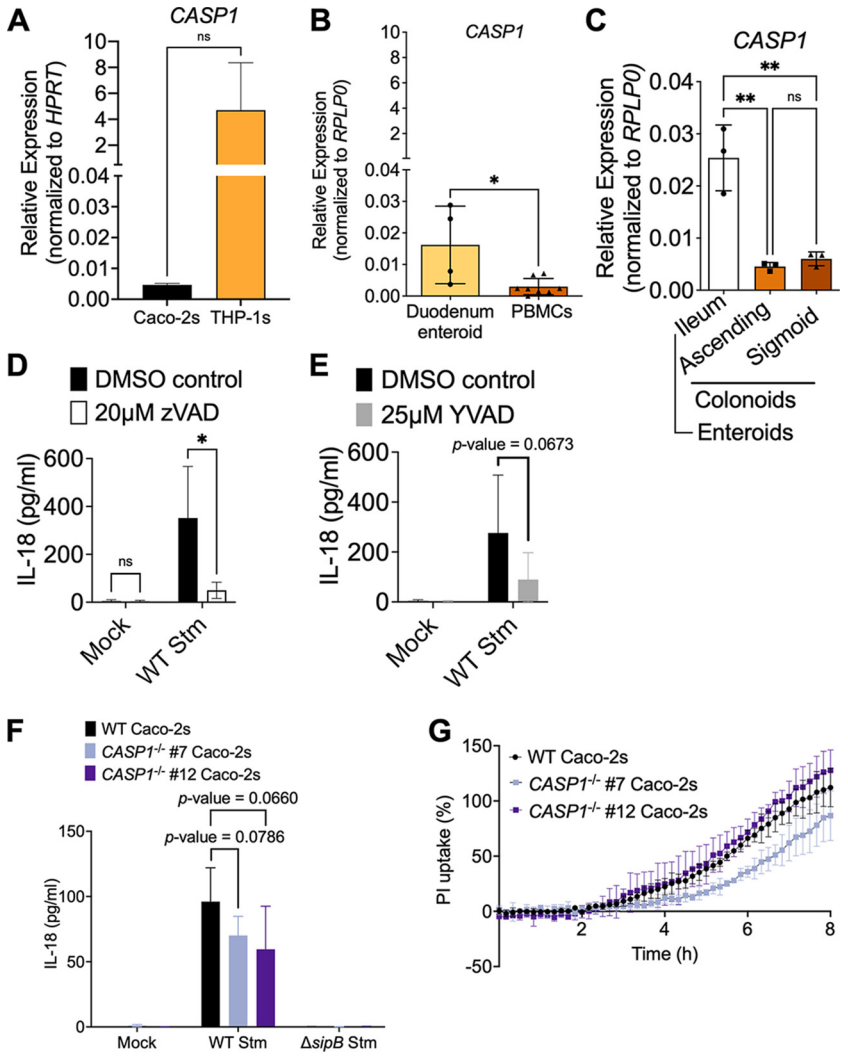

FIG 6 Caspase-1 is not a major contributor to inflammasome responses to *Salmonella* in human intestinal epithelial cells. (A to C) Relative mRNA expression of *CASP1* compared to the housekeeping control *HPRT* or *RPLP0* as measured by RT-qPCR in Caco-2 cells, THP-1 macrophages, human peripheral blood mononuclear cells (PBMCs), and human small intestinal enteroids or colonoids. (D and E) WT Caco-2 cells were primed with 400 ng/mL of Pam3CSK4 for 3 h. One hour prior to infection, cells were treated with 20 μM pan-caspase inhibitor Z-VAD(OMe)-FMK, 25 μM caspase-1 inhibitor Ac-YVAD-cmk, or DMSO as a vehicle control. Cells were then infected with PBS (Mock), WT *S.* Typhimurium, or Δ*sipB S.* Typhimurium for 6 h. Release of IL-18 into the supernatant was measured by ELISA. (F and G) WT or two independent single-cell clones of *CASP1*$^{-/-}$ Caco-2 cells were infected with PBS (Mock), WT *S.* Typhimurium, or Δ*sipB S.* Typhimurium. (F) Release of IL-18 into the supernatant was measured by ELISA at 6 hpi. (G) Cell death was measured as percent uptake of propidium iodide, normalized to cells treated with 1% Triton. ns, not significant; *, $P < 0.05$; **, $P < 0.01$, by unpaired *t* test (A and B), Tukey's multiple-comparison test (C), Šídák's multiple-comparison test (D), paired *t* test (E), or Dunnett's multiple-comparison test (F). Shown are the averages and error bars representing the standard deviation for at least three pooled independent experiments.

observed that expression of *CASP1* mRNA in Caco-2 cells is lower than that in THP-1 macrophages (Fig. 6A), in agreement with previous findings (53). However, *CASP1* expression in Caco-2 cells is still higher than expression of the other inflammasome genes we have assessed so far (compare axes in Fig. 6A to Fig. 3 and 5B and F). Indeed, expression of *CASP1* is higher in intestinal enteroids and colonoids than in human PBMCs, and *CASP1* expression is higher than expression of other inflammasome-related genes (compare axes in Fig. 6B and C to Fig. 3C to F) (55). We thus asked whether caspase-1 contributes to inflammasome responses against *Salmonella* in IECs.

We first tested the contribution of caspase activity to inflammasome responses during *Salmonella* infection in human IECs. We pretreated unprimed and Pam3CSK4-primed Caco-2 cells with pharmacological inhibitors targeting caspases of interest (ZVAD [pan-caspase inhibitor] or YVAD [inhibitor for caspase-1]). Under both unprimed and primed conditions, DMSO-treated cells infected with WT Stm released IL-18, whereas cells treated with ZVAD or YVAD had a significant defect in IL-18 release (Fig. 6D and E; Fig. S5B), corroborating previous findings (53, 55). Treatment with ZVAD, the pan-caspase inhibitor, resulted in a lower level of IL-18 release than did treatment with the caspase-1 inhibitor YVAD, suggesting that in addition to caspase-1, other caspases also contribute to inflammasome responses during *Salmonella* infection of human IECs (Fig. 6D and E; Fig. S5B). As expected, cells infected with Δ*sipB* Stm did not induce inflammasome activation regardless of inhibitor treatment (Fig. 6D and E; Fig. S5B). These results suggest that caspase-1 and possibly additional caspases contribute to inflammasome responses to *Salmonella* in human IECs.

Since pharmacological inhibitors preferentially targeting individual caspases can cross-react with other caspases, we used CRISPR/Cas9 to disrupt *CASP1* in Caco-2 cells (Fig. S8 and S9). We sequenced and validated two independent single-cell clones of *CASP1*$^{-/-}$ Caco-2 cells (*CASP1*$^{-/-}$ #7, *CASP1*$^{-/-}$ #12) (Fig. S8 and S9). All mutations resulted in a premature stop codon (Fig. S8 and S9). We further confirmed decreased mRNA expression of *CASP1* in *CASP1*$^{-/-}$ #7 Caco-2 cells (Fig. S8B). We were unable to detect caspase-1 protein expression in both WT Caco-2 cells and *CASP1*$^{-/-}$ #7 Caco-2 cells by Western blotting (Fig. S8C), likely due to low caspase-1 expression in Caco-2 cells (53, 55).

We infected WT or *CASP1*$^{-/-}$ Caco-2 cells with WT or Δ*sipB* Stm and assayed for subsequent inflammasome activation (Fig. 6F and G). As expected, WT Caco-2 cells infected with WT Stm released significant levels of IL-18 and underwent cell death (Fig. 6F and G). This response was dependent on the presence of the SPI-1 T3SS, as cells infected with Δ*sipB* Stm failed to undergo inflammasome activation (Fig. 6F). Consistent with YVAD inhibitor treatment, *CASP1*$^{-/-}$ Caco-2 cells infected with WT Stm showed a slight decrease in IL-18 release at 6 hpi that approached statistical significance (Fig. 6F). There was also a slight delay in uptake of PI in *CASP1*$^{-/-}$ #7 Caco-2 cells relative to WT Caco-2 cells (Fig. 6F). In contrast, WT and KO Caco-2 cells released similar levels of the inflammasome-independent cytokine IL-8 (Fig. S10). Overall, these data indicate that caspase-1 may partially contribute to inflammasome responses to *Salmonella* infection in human IECs but is not required.

Caspase-4 is required for inflammasome responses to *Salmonella* in human intestinal epithelial cells. In mice, in addition to caspase-1, a second caspase, caspase-11, responds to *Salmonella* infection (54, 56). Caspase-11 and its human orthologs caspases-4/5 detect cytosolic LPS and form the noncanonical inflammasome (46, 47). Caspases-4/5 are important for inflammasome responses to *Salmonella* infection of human IECs, specifically when *Salmonella* escapes to the cytoplasm from its *Salmonella*-containing vacuole (SCV) (53, 55). To test the contribution of the caspase-4/5 inflammasome in our model, we transfected unprimed and Pam3CSK4-primed Caco-2 cells with small interfering RNAs (siRNAs) targeting *CASP4*, *CASP5*, or both. We then infected the cells with WT or Δ*sipB* Stm and assayed for inflammasome activation by measuring IL-18 release (Fig. 7A and Fig. S11A). As expected, under both unprimed and primed conditions, cells treated with a control scrambled siRNA exhibited IL-18 secretion upon infection with WT Stm but failed to undergo IL-18 secretion when infected with Δ*sipB* Stm (Fig. 7A and Fig. S11A). However, knockdown of *CASP4*, either alone or in conjunction with *CASP5*, resulted in nearly complete abrogation of IL-18 secretion in cells infected with WT Stm compared to control siRNA-treated cells, suggesting that caspase-4 is required for inflammasome activation (Fig. 7A and Fig. S11A), in agreement with previous findings (53, 55). Knockdown of *CASP5* alone resulted in a partial and significant decrease in IL-18 secretion, indicating that while caspase-5 may be playing a role, it is not absolutely required (Fig. 7A and Fig. S11A). In both primed and unprimed cells, we observed moderately high siRNA-mediated knockdown

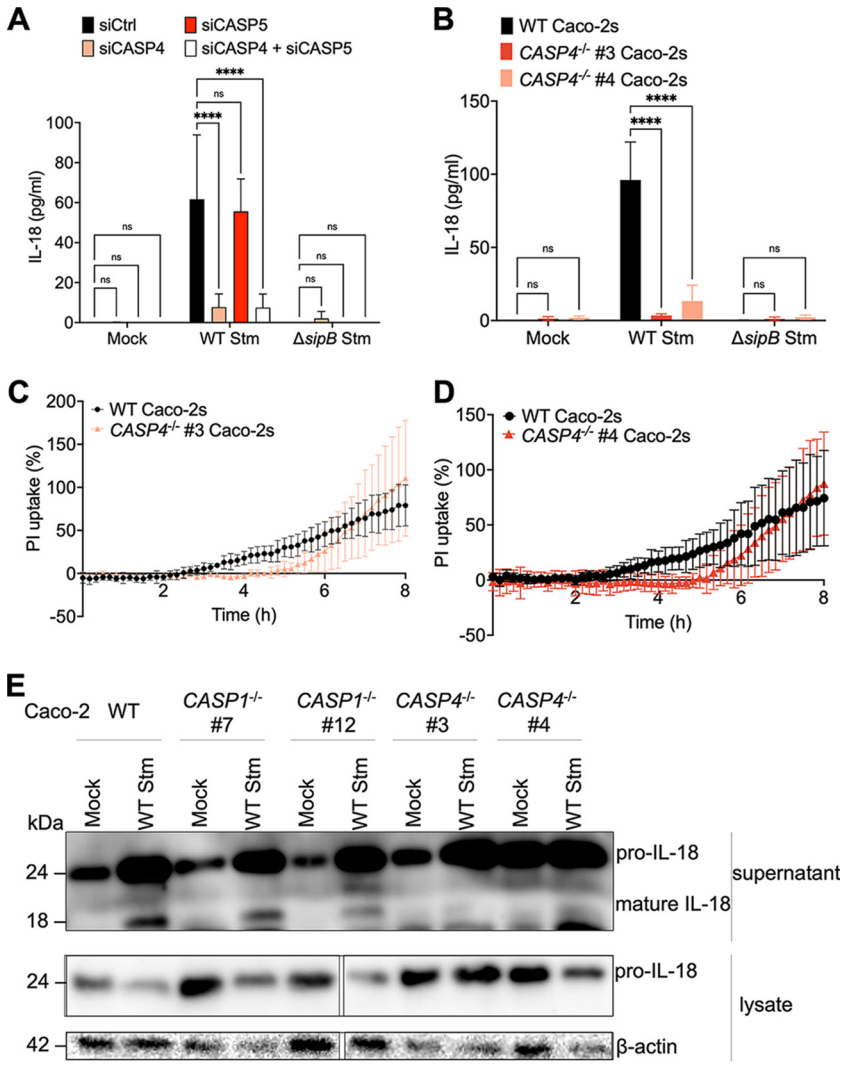

FIG 7 Caspase-4 is required for inflammasome responses to *Salmonella* in human intestinal epithelial cells. (A) WT Caco-2 cells were treated with siRNA targeting *CASP4* or *CASP5* or a control scrambled siRNA for 72 h. Cells were primed with 400 ng/mL of Pam3CSK4 for 3 h. Cells were then infected with PBS (Mock), WT *S.* Typhimurium, or Δ*sipB* S. Typhimurium for 6 h. Release of IL-18 was measured by ELISA. (B to E) WT or two independent clones of *CASP4*$^{-/-}$ or *CASP1*$^{-/-}$ (E only) Caco-2 cells were infected with PBS (Mock), WT *S.* Typhimurium, or Δ*sipB S.* Typhimurium for 6 h. (B) Release of IL-18 into the supernatant was measured by ELISA at 6 hpi. (C and D) Cell death was measured as percent uptake of propidium iodide, normalized to cells treated with 1% Triton. (E) Lysates and supernatants collected 6 hpi were immunoblotted for IL-18 and *β*-actin. ns, not significant; ****, $P < 0.0001$, by Dunnett's multiple-comparison test. Shown are the averages and error bars representing the standard deviation for at least three pooled independent experiments.

efficiencies (66 to 84%) (Fig. S11B and C). Release of the inflammasome-independent cytokine IL-8 was comparable across conditions (Fig. S11D).

To definitively test the requirement of caspase-4 in early inflammasome responses to SPI-1-expressing *Salmonella*, we disrupted *CASP4* in Caco-2 cells using CRISPR/Cas9 (Fig. S12 and S13). We sequenced and validated two independent single-cell clones of *CASP4*$^{-/-}$ Caco-2 cells (*CASP4*$^{-/-}$ #3, *CASP4*$^{-/-}$ #4) (Fig. S12). All mutations resulted in a premature stop codon (Fig. S12). Both clones exhibited decreased mRNA expression of *CASP4* and no protein expression relative to WT Caco-2 cells (Fig. S13).

We then infected WT or *CASP4*$^{-/-}$ Caco-2 cells with WT or Δ*sipB* Stm and assayed for subsequent IL-18 secretion and cell death as readouts for inflammasome activation (Fig. 7B to E). As expected, WT Caco-2 cells infected with WT Stm released significant levels of cleaved IL-18 and underwent cell death (Fig. 7B to E), whereas cells infected

with Δ*sipB* Stm failed to release IL-18 (Fig. 7B). Consistent with our findings with siRNA knockdown of *CASP4*, *CASP4*$^{-/-}$ Caco-2 cells infected with WT Stm failed to release IL-18 and exhibited a delay in PI uptake at 4 to 6 hpi (Fig. 7B to E). Interestingly, *CASP4*$^{-/-}$ Caco-2 cells began to undergo cell death later in infection, suggesting that while caspase-4 is required for inducing cell death early in infection, it may not be absolutely required at later time points of infection. Like WT cells, *CASP4*$^{-/-}$ cells still released substantial levels of the inflammasome-independent cytokine IL-8 following infection (Fig. S11E).

Inflammasome activation promotes control of infection through multiple mechanisms. In mice, *Salmonella* infection is controlled through restriction of bacterial replication, extrusion of infected cells, and prevention of systemic dissemination of *Salmonella* (27–29, 44, 53, 76–78). Human IECs restrict bacterial replication and undergo extrusion through caspase-4-dependent mechanisms (53, 55, 79). To test if caspase-4 plays a similar role in restricting bacterial replication in our model, we infected WT and *CASP4*$^{-/-}$ Caco-2 cells with WT Stm and measured the bacterial CFU at various time points postinfection to assay bacterial burdens (Fig. S11F). Consistent with previous findings (53, 55), we found the bacterial burdens to be lower in WT Caco-2 cells than in *CASP4*$^{-/-}$ Caco-2 cells at each time point assayed, suggesting that caspase-4 plays a critical role in controlling bacterial burdens in human IECs. Collectively, these data are in agreement with previous findings (53, 55) that caspase-4 is required for inflammasome activation and subsequent bacterial restriction in response to SPI-1-expressing *Salmonella* infection in human IECs.

DISCUSSION

In this study, we have demonstrated that Caco-2 cells undergo inflammasome activation in response to *Salmonella* infection in an SPI-1 T3SS-dependent manner and that GSDMD-mediated pore formation is required for release of IL-18 and cell death (Fig. 1; see also Fig. S1 in the supplemental material). Unexpectedly, however, delivery of the individual NAIP ligands derived from the T3SS or flagellin was not sufficient to induce inflammasome activation in Caco-2 cells or intestinal enteroids or colonoids (Fig. 2 and Fig. S2 and S3). Additionally, we found that neither the NAIP nor NLRP3 canonical inflammasomes (Fig. 4 and 5 and Fig. S4 and S5A) are required for inflammasome responses to *Salmonella* infection, which may be due to low mRNA expression of *NAIP*, *NLRC4*, and *NLRP3* in human IECs and human intestinal enteroids and colonoids (Fig. 3 and 5). Moreover, we found that ASC, a shared adaptor protein that several inflammasomes use to recruit caspases, is also not required for inflammasome activation (Fig. 5 and Fig. S6 and S7), suggesting that inflammasomes that require ASC for their function are likely not playing a role in human IECs during *Salmonella* infection. We found that caspase-1 is not required for inflammasome activation in human IECs, although it may partially contribute (Fig. 6 and Fig. S5B and S8 to S10). Finally, we found that caspase-4 is necessary for inflammasome responses to SPI-1-expressing *Salmonella* and contributes to restriction of intracellular *Salmonella* (Fig. 7 and Fig. S11 to S13), in agreement with previous findings (53, 55).

NAIP/NLRC4 inflammasome activation in IECs is both necessary and sufficient for control of *Salmonella* infection in mice (27–29). It was therefore surprising that the NAIP/NLRC4 inflammasome does not seem to be functional in IECs (Fig. 2 and Fig. S2 and S3) and that it was dispensable in human IECs during *Salmonella* infection (Fig. 4 and Fig. S4). Perhaps a lack of robust NAIP/NLRC4 inflammasome responses in human IECs partially underlies why some intestinal bacterial pathogens with T3SS, such as *Shigella flexneri*, enterohemorrhagic *Escherichia coli*, and enteropathogenic *E. coli*, can cause disease in humans but not mice. Indeed, mice are normally resistant to *S. flexneri*, but mice lacking the NAIP/NLRC4 inflammasome can be robustly colonized by *S. flexneri* and develop a disease resembling human shigellosis (80).

The lack of NAIP activation may be due to low expression of *NAIP* and *NLRC4* mRNA in human IEC lines as well as human small intestinal enteroids and colonoids (Fig. 3). Single-cell transcriptome analysis of intestinal cells from human donors indicates that only a small

number of intestinal cells express *NAIP* at detectable levels, while *NLRC4* expression is below the limit of detection (81). However, it is possible that a population of IECs exist that express *NAIP* and *NLRC4* at low levels that may be below the limit of detection of single-cell transcriptome analysis but that may be sufficient to activate the inflammasome in response to infection under physiological conditions. Indeed, single-cell transcriptome analysis of murine small intestines reveals that expression of *NLRC4* is also very low in murine IECs (82), but murine IECs undergo robust NAIP/NLRC4 inflammasome activation despite this relatively low expression (27–29). Despite our findings that NAIP is dispensable in Caco-2 cells to respond to *Salmonella* infection and human intestinal organoids enteroids and colonoids do not mount NAIP inflammasome responses to T3SS ligands, it is possible that under other physiological conditions or during an *in vivo* setting, the NAIP/NLRC4 inflammasome still plays an important role during bacterial infection or disease pathogenesis. Indeed, human patients with activating NLRC4 mutations exhibit gastrointestinal symptoms (35–38), suggesting that this inflammasome may play a critical, but yet unexplored, role in the human gastrointestinal tract.

A recent study comparing inflammasome responses of human and murine IECs during *Salmonella* infection reported that while caspase-1 was required for inflammasome activation in murine IECs, it was dispensable in inflammasome responses to *Salmonella* in human IECs (55). We also found caspase-1 was not required for inflammasome activation in IECs infected with *Salmonella*, but we observed that caspase-1 may partially contribute to inflammasome responses (Fig. 6 and Fig. S5B and S8 to S10). Differences in our experimental conditions likely explain this slight difference in findings. Our *Salmonella* was grown under SPI-1-inducing conditions, and we assayed for inflammasome activation at a slightly earlier time point. Perhaps caspase-1 plays more of a role in early inflammasome responses to SPI-1-expressing *Salmonella* in human IECs, and it is less important at later time points when *Salmonella* has shifted to expressing its SPI-2 T3SS.

Whether there are host sensors that act upstream of caspase activation in human IECs during *Salmonella* infection remains unknown. Humans have 22 NLRs that could serve as potential cytosolic sensors of bacterial structures and activity (83). Most of these NLRs do not contain a CARD domain, and they would therefore require an adaptor protein such as ASC to recruit caspases. Given our finding that ASC is not required for inflammasome activation in human IECs (Fig. 5 and Fig. S6 and S7), it is likely that if a putative host sensor exists, it would contain its own CARD domain. Alternatively, it may interact with a different CARD domain-containing adaptor protein other than ASC, or in the case of caspase-4 or -5, the caspase may act as the sensor itself and directly detect bacterial LPS. Future studies will focus on identifying host factors that caspases interact with during *Salmonella* infection of human IECs.

In agreement with previous studies (53, 55), we found caspase-4 to be required for inflammasome activation in response to *Salmonella* in human IECs (Fig. 7 and Fig. S11 to S13). Importantly, caspase-4 restricts bacterial replication (Fig. S11F), and it specifically restricts replication of a subpopulation of cytosolic *Salmonella* (53, 55). It is unclear whether *Salmonella*'s access into the cytosol is mediated by the bacteria or host or both, and the mechanisms by which cytosolic populations of *Salmonella* arise and how they influence inflammasome activation in human IECs are also unclear.

In mice, inflammasome activation mediates control of *Salmonella* by restricting bacterial replication, causing extrusion of infected cells, and preventing systemic dissemination of *Salmonella* (27, 29, 44, 53, 76–78). Human IECs restrict bacterial replication and undergo extrusion through caspase-4-dependent mechanisms (53, 55). However, it remains unknown if inflammasome activation results in additional mechanisms that contribute to control of infection. In mice, IL-18 release during *Salmonella* infection recruits natural killer (NK) cells that are critical for early mucosal inflammatory responses (84). We observe robust release of the inflammasome-dependent cytokine IL-18, but the downstream role of this cytokine in the human intestine during *Salmonella* infection has not been explored. Additionally, while our study focused exclusively on *Salmonella* infection, it is worth

exploring if enteric pathogens with lifestyles similar to *Salmonella*, such as *Shigella*, or ones with different lifestyles, such as the extracellular pathogen *Yersinia*, elicit similar responses. Future studies that interrogate downstream consequences of inflammasome activation in human IECs in response to various enteric pathogens could shed light on human mucosal inflammatory responses to bacterial pathogens.

Overall, our data indicate that *Salmonella* infection of human IECs triggers inflammasome pathways that are distinct from those activated in murine IECs. Pathways that are activated in murine IECs or human macrophages and important for control of infection, such as the NAIP and NLRP3 inflammasomes, were unexpectedly not required for inflammasome responses in human IECs under the conditions we investigated. Instead, caspase-4 was the primary mediator of inflammasome responses in human IECs during *Salmonella* infection. These findings indicate how inflammasome responses can vary between mice and humans as well as in different cell types. Our findings provide a foundation for future studies aimed at uncovering the relative contribution of different caspases and the downstream responses that they mediate in human IECs in response to *Salmonella* and other pathogens.

MATERIALS AND METHODS

Ethics statement. All studies involving human peripheral blood mononuclear cells (PBMCs), human intestinal enteroids, and human intestinal organoids were performed in compliance with the requirements of the U.S. Department of Health and Human Services and the principles expressed in the Declaration of Helsinki. Human PBMCs, intestinal enteroids, and colonoids are all considered to be a secondary use of deidentified human specimens and are exempt via Title 55 Part 46, Subpart A of 46.101 (b) of the Code of Federal Regulations. For experiments performed with human organoids, biopsy specimens were collected from consenting adult patients at Oregon Health & Science University (OHSU), with the approval of the OHSU Institutional Review Board (protocol no. 00020848). All experiments performed with murine enteroids were done so in compliance with the regulatory standards of, and were approved by, the Oregon Health & Science University Institutional Animal Care and Use Committee.

Bacterial strains and growth conditions. *Salmonella enterica* serovar Typhimurium SL1344 WT and Δ*sipB* (85) isogenic strains were routinely grown shaking overnight at 37°C in Luria-Bertani (LB) broth with streptomycin (100 μg/mL). Cells were infected with *Salmonella* grown under SPI-1-inducing conditions (86).

Listeria monocytogenes WT and isogenic strains on the 10403S background were routinely grown shaking overnight at 30°C in brain heart infusion (BHI) broth (68). *Salmonella* Typhimurium ligand PrgJ or PrgI was translationally fused to the truncated N terminus of ActA and under the control of the *actA* promoter (68).

Bacterial infections. Where indicated, cells were primed with 100 ng/mL or 400 ng/mL of Pam3CSK4 (InvivoGen) for 3 h prior to infection. To induce SPI-1 expression, overnight cultures of *Salmonella* were diluted into LB broth containing 300 mM NaCl and grown for 3 h standing at 37°C (86). Overnight cultures of *Listeria* were diluted and grown shaking for 3 h in BHI at 30°C. All cultures were pelleted at 6,010 $\times$ *g* for 3 min, washed once with phosphate-buffered saline (PBS), and resuspended in PBS. Cells were infected with *Salmonella* at a multiplicity of infection (MOI) of 60 or *Listeria* at the indicated MOI for each experiment in the figure legend. Infected cells were centrifuged at 290 $\times$ *g* for 10 min and incubated at 37°C. At 1 h postinfection, cells were treated with 100 ng/mL or 50 ng/mL of gentamicin to kill any extracellular *Salmonella* or *Listeria,* respectively. Infections proceeded at 37°C for the indicated length of time for each experiment. Control cells were mock infected with PBS for all experiments.

Cell culture of intestinal epithelial cell lines. All cell lines were obtained from the American Type Culture Collection (ATCC). Caco-2 cells (HTB-37; ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 μg/mL streptomycin. T84 cells (CCL-248; ATCC) were maintained in DMEM–F-12 supplemented with 5% (vol/vol) heat-inactivated FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin. THP-1 cells (TIB-202; ATCC) were maintained in RPMI supplemented with 10% (vol/vol) heat-inactivated FBS, 0.05 nM β-mercaptoethanol, 100 IU/mL penicillin, and 100 μg/mL streptomycin. All cell lines were grown at 37°C in a humidified incubator with 5% CO_2.

One day prior to infection or treatment, Caco-2 or T84 cells were dissociated with 0.25% trypsin-EDTA (Gibco) diluted 1:1 with 1$\times$ PBS. Cells were incubated with trypsin at 37°C for 15 min, after which the trypsin was neutralized with serum-containing medium. Cells were replated in medium without antibiotics in a 24-well plate at a concentration of 3 $\times$ 10^5 cells/well. Where indicated, cells were primed with 100 ng/mL or 400 ng/mL Pam3CSK4 (InvivoGen) for 3 h prior to bacterial infections.

THP-1 cells were replated in medium without antibiotics in a 48-well plate at a concentration of 2 $\times$ 10^5 cells/well and incubated with phorbol 12-myristate 13-acetate (PMA) for 24 h to allow differentiation into macrophages. Macrophage cell lysates were collected to harvest RNA.

Culture of intestinal enteroids and colonoids in spheroid culture. Spheroids derived from human duodenum or colon used in Fig. 2, Fig. 3C and D, and Fig. 6B were kindly provided by Jared Fisher at Oregon Health & Science University. Enteroids and colonoids in Fig. 2, Fig. 3C and D, and Fig. 6B were derived from 2 separate human donors (#1 and 2), and enteroids and colonoids in Fig. 3E and F and

Fig. 6C were from a third donor. Murine enteroids were derived from one C57BL/6J mouse of unrecorded sex. Human and murine enteroids were generated and cultivated in special conditioned medium as described previously (87, 88).

Briefly, for human organoids, biopsy specimens were collected from consenting adult patients at OHSU and placed in wash medium (Advanced DMEM–F-12 [Gibco, catalog no. 12634010] plus 10% fetal bovine serum [FBS], 1 U/mL penicillin-streptomycin [P/@] with glutamate [L-glu], and 1 mM HEPES) on ice. This study was approved by the OHSU Institutional Review Board (protocol no. 00020848). Biopsy specimens were minced in 2 mg/mL collagenase type I solution (Gibco, catalog no. 17018029) and digested for 20 min at 37°C with trituration.

For murine organoids, small intestinal tissue was scraped with a glass slide to remove villi and incubated with mild shaking for 30 min at 4°C in Ca/Mg-free PBS containing 2 mM EDTA. PBS-EDTA was decanted, and tissue was shaken vigorously in cold PBS to remove crypts.

For both protocols, digested material was filtered (70 μm) into bovine serum albumin (BSA)-coated conical tubes, diluted with wash medium, and centrifuged at 500 relative centrifugal force (RCF) for 5 min. Crypts were resuspended in wash medium and centrifuged at 500 RCF. Purified crypts were then resuspended in Matrigel (Corning, catalog no. 354234) and plated in 15-μL droplets in 24-well plates and maintained in 0.5 mL conditioned medium (CM) from L-WRN cells (ATCC catalog no. CRL-3276), which contains Wnt3a, R-spondin3, and Noggin. Inhibitors of ROCK (Y-27632; SelleckChem) and transforming growth factor beta (TGF-β) (SB-431542; SelleckChem) were added to the culture medium to promote cell survival. Medium was replaced every 2 days. Organoids were passaged when they became large and dense, approximately every 4 to 8 days.

ELISAs. Supernatants harvest from infected cells were assayed using enzyme-linked immunosorbent assay (ELISA) kits for human IL-18 (R&D Systems) and IL-8 (R&D Systems).

Immunoblot analysis. Cell lysates were harvested for immunoblot analysis by adding 1× SDS-PAGE sample buffer to cells following infection. Cells were incubated and infected in serum-free medium to collect supernatant samples. Supernatant samples were centrifuged at 200 × *g* to pellet any cell debris. The supernatants were then treated with trichloroacetic acid (TCA) (25 μL TCA per 500 μL supernatant) overnight at 4°C. The next day, the samples were centrifuged at maximum speed (15,871 × *g*) for 15 min at 4°C. Precipitated supernatant pellets were washed with ice-cold acetone, centrifuged at maximum speed (15,871 × *g*) for 10 min at 4°C, and resuspended in 1× SDS-PAGE sample buffer. All protein samples (lysates and supernatants) were boiled for 5 min. Samples were separated by SDS-PAGE on a 12% (vol/vol) acrylamide gel and transferred to polyvinylidene difluoride (PVDF) Immobilon-P membranes (Millipore). Primary antibodies specific for human IL-18 (MBL International PM014) and β-actin (4967L; Cell Signaling) and horseradish peroxidase (HRP)-conjugated secondary antibody anti-rabbit IgG (7074S; Cell Signaling) were used. Enhanced chemiluminescence (ECL) Western blotting substrate or SuperSignal West Femto (both from Pierce Thermo Scientific) was used as the HRP substrate for detection.

PI uptake assay. Caco-2 cells (7.5×10^4 per well) were plated in a black, flat-bottom 96-well plate (CellStar) in PI uptake medium containing 1× Hanks balanced salt solution (HBSS) without phenol red, 20 mM HEPES, and 10% (vol/vol) heat-inactivated FBS. Cells were infected at an MOI of 60, and control wells were treated with 1% Triton. After infection, cells were centrifuged at 290 × *g* for 10 min following infection. A 5 μM concentration of propidium iodide (P3566; Invitrogen) diluted in PI uptake medium was added to the cells. The plate was sealed with adhesive optical plate sealing film (Microseal; Bio-Rad) and placed in a Synergy H1 microplate reader (BioTek) preheated to 37°C. PI fluorescence was measured every 10 min for the indicated number of hours.

Anthrax toxin-mediated delivery of bacterial ligands into enteroids and colonoids. Enteroids and colonoids from one donor each were seeded in 96-well plates with transparent bottoms and opaque walls in 5 μL Matrigel domes. After 2 days in enteroid medium, enteroids and colonoids were grown for an additional 3 days in differentiation medium: DMEM–F-12 supplemented with 20% murine R-Spondin1 supernatant, 10% murine Noggin supernatant, 50 ng/mL recombinant murine epidermal growth factor (EGF) (Fisher Scientific), 1× P/S, 1× L-Glu, 10 mM HEPES (HiMedia), 1× N2 supplement (Life Technologies), 1× B27 supplement (Life Technologies), and 1 mM *N*-acetylcysteine (Fisher Scientific). A 5 μM concentration of the gamma-secretase inhibitor DAPT was added for the last 24 h. Differentiated enteroids and colonoids were then treated with 16 μg/mL PA and 8 μg/mL LFn-FlaA or 0.1 μg/mL LFn-PrgJ in differentiation medium containing 10 μg/mL propidium iodide for 4 h. Total lysis wells were treated with 1% Triton.

Expression of inflammasome genes in human small intestinal enteroids and colonoids. To analyze inflammasome expression, enteroids and colonoids from donor 3 were cultured as described above (Fig. 3E and F and Fig. 6C), and enteroids from donor 1 were cultured in differentiation medium (Fig. 3C and D and Fig. 6B): DMEM–F-12 supplemented with 20% supernatant from R-Spondin1-expressing L-cells, 10% supernatant from Noggin-expressing cells, 50 ng/mL recombinant murine EGF (Fisher Scientific), 1× P/S, 1× L-Glu, 10 mM HEPES (HiMedia), 1× N2 (Life Technologies), 1× B27 (Life Technologies), 1 mM *N*-acetylcysteine (Fisher Scientific), and 5 μM DAPT. After 4 days of incubation at 37°C, Matrigel domes were dissolved in PBS-EDTA (5 mM) for 1 h at 4°C on an orbital shaker. After centrifugation at 300 × *g* for 5 min at 4°C, cells were harvested in TRIzol reagent (Thermo Fisher Scientific) or ZR-Quick viral RNA kit buffer (Zymo Research) for mRNA analysis.

Isolation of PBMCs. To compare expression levels of inflammasome components in human IECs with those in immune cells, PBMC-derived cDNA was kindly provided by William Messer at Oregon Health & Science University. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated using density gradient centrifugation. After overlay of Lymphoprep (Alere Technologies AS) with blood mixed 1:2 with 1× PBS (pH 7.4, Gibco), the sample was centrifuged at 800 × *g* for 20 min at room temperature (RT). Residual erythrocytes (RBCs) were lysed with 1× RBC lysis buffer (10×, BioLegend), followed by

three washing steps with fresh PBS for 10 min at 250 × *g*. Subsequently, cells were harvested in TRIzol reagent (Thermo Fisher Scientific) for mRNA analysis.

RNA extraction, cDNA synthesis, and real-time quantitative PCR (RT-qPCR) of enteroid, colonoid, and PBMC samples. After thawing the TRIzol samples, chloroform was added and the tubes were centrifuged for 15 min at 12,000 × *g* at 4°C. The aqueous phase was transferred to a new tube containing linear polyacrylamide (Gene-Elute LPA; Sigma). To allow RNA precipitation, the samples were incubated with isopropanol for 10 min and subsequently centrifuged for 10 min at 12,000 × *g*. After aspiration of the supernatant, the RNA pellet was washed once with 75% ethanol for 5 min at 7,500 × *g* at 4°C. The supernatant was aspirated, and the dried pellet was resuspended in molecular biology-grade water (Corning). For RNA used in Fig. 3E and F, RNA was isolated using the ZR Quick-Viral RNA or ZR Quick-Viral RNA 96 kit (Zymo Research). DNA contamination was removed using the DNA-free kit (Life Technologies). After determination of RNA content and quality (260/280 and 260/230 ratios), 1 μg of RNA was reversely transcribed into cDNA. Reaction steps were performed in a Bio-Rad T100 cycler. First, residual DNA was removed using RQ1 RNase-free DNase (Promega) in RQ1 DNase 1× reaction buffer (Promega) for 30 min at 37°C. After stopping the reaction with RQ1 DNase stop solution (Promega) for 10 min at 65°C, oligo(dT)s (Sigma) and deoxynucleoside triphosphates (dNTPs) (Sigma) were added for 5 min at 65°C. Reverse transcription was performed with SuperScript IV reverse transcriptase (Invitrogen), 5 mM dithiothreitol (DTT), and SuperScript IV reaction buffer (Invitrogen) for 10 min at 50 to 55°C. Subsequently, the enzyme was inactivated at 80°C for 10 min.

To analyze RNA expression, cDNA was mixed with 10 μM forward and reverse primers and PowerUp SYBR Green Master Mix (Applied Biosystems) according to manufacturer's instructions. The following primers were used: *RPLP0*, forward, 5′-GGCACCATTGAAATCCTGAGTGATGTG-3′; reverse, 5′-TTGCGGACAC CCTCCAGGAAG-3′; *NAIP*, forward, 5′-AAGCATCCGCCCAGCTCTTGA-3′; reverse, 5′-TATTGCCCTCCAGATCC ACAGACAGTTC-3′; *NLRC4*, forward, 5′-CATAGTCAAGTCTCTGTCAAGTGAACCCTGT-3′; reverse, 5′-GCTGT TCTAGCACGTTCATCCTGTCG-3′; *CASP1*, forward, 5′-GAGGCATTTGCACACCGCCC-3′; reverse, 5′-GGATCTC TTCACTTCCTGCCCACA-3′.

Amplification was analyzed in real time with StepOne Software v2.3. In brief, samples were incubated for 10 min at 95°C, followed by 40 cycles of heating to 95°C for 15 s and cooling to 60°C for 1 min. To monitor specificity of the run, the melt curves were determined by keeping the samples at 95°C for 15 s, cooling to 60°C for 1 min, and then increasing the temperature every 15 s by 0.3°C up to 95°C. Expression levels relative to the housekeeping gene (*RPLP0*) were calculated using the formula $x = 2^{-\Delta CT}$.

RNA extraction, cDNA synthesis, and RT-qPCR of Caco-2, T84, and THP-1 samples. RNA was isolated using the RNeasy Plus minikit (Qiagen) following the manufacturer's instructions. Cells were lysed in 350 μL RLT buffer with β-mercaptoethanol and centrifuged through a QIAshredder spin column (Qiagen). cDNA was synthesized from isolated RNA using SuperScript II reverse transcriptase (Invitrogen) following the manufacturer's protocol. Quantitative PCR was conducted with the CFX96 real-time system from Bio-Rad using the SsoFast EvaGreen Supermix with Low ROX (Bio-Rad). To calculate relative gene expression, mRNA levels of target genes were normalized to housekeeping gene *HPRT* and the formula $x = 2^{-\Delta CT}$ was used. The following primers from PrimerBank (PrimerBank identification listed within parentheses) were used (89–91): *HPRT* (164518913c1), forward, 5′-CCTGGCGTCGTGATTAGTGAT-3′; reverse, 5′-AGACGTTCAGTCCTGTCCATAA-3′; *NAIP* (119393877c3), forward, 5′-CCCATTAGACGATCA CACCAGA-3′; reverse, 5′-GGAGTCACTTCCGCAGAGG-3′; *NLRC4* (312433959c2), forward, 5′-TGCATCATT GAAGGGGAATCTG-3′; reverse, 5′-GATTGTGCCAGGTATATCCAGG-3′; *NLRP3* (208879435c1), forward, 5′-GATCTTCGCTGCGATCAACAG-3′; reverse, 5′-CGTGCATTATCTGAACCCCAC-3′; *PYCARD* (313482805c1), forward, 5′-TGGATGCTCTGTACGGGAAG-3′; reverse, 5′-CCAGGCTGGTGTGAAACTGAA-3′; *CASP1* (380254454c1), forward, 5′-TTTCCGCAAGGTTCGATTTTCA-3′; reverse, 5′-GGCATCTGCGCTCTACCATC-3′.

Inhibitor experiments. Cells were treated 1 h prior to infection at the indicated concentrations with the following inhibitors: various concentrations of MCC950 (Sigma-Aldrich; PZ0280), 20 μM pan-caspase inhibitor Z-VAD(OMe)-FMK (SM Biochemicals; SMFMK001), 25 μM caspase-1 inhibitor Ac-YVAD-cmk (Sigma-Aldrich; SML0429), and 30 μM disulfiram (Sigma).

siRNA-mediated gene knockdown. The following Silencer Select siRNA oligonucleotides were purchased from Ambion (Life Technologies): *CASP4* (s2412), *CASP5* (s2417), and two Silencer Select negative-control siRNAs (Silencer Select negative control no. 1 siRNA and Silencer Select negative control no. 2 siRNA). Three days before infection, 30 nM siRNA was transfected into Caco-2 cells using Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific) following the manufacturer's protocol.

Statistical analysis. Prism 9.3.1 (GraphPad Software) was used to graph all data and for all statistical analyses. Statistical significance for experiments was determined using the appropriate test and is indicated in each figure legend. Differences were considered statistically significant if the *P* value was <0.05.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 4.4 MB.

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