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The *Salmonella* pathogenicity island 2 subverts human NLRP3 and NLRC4 inflammasome responses

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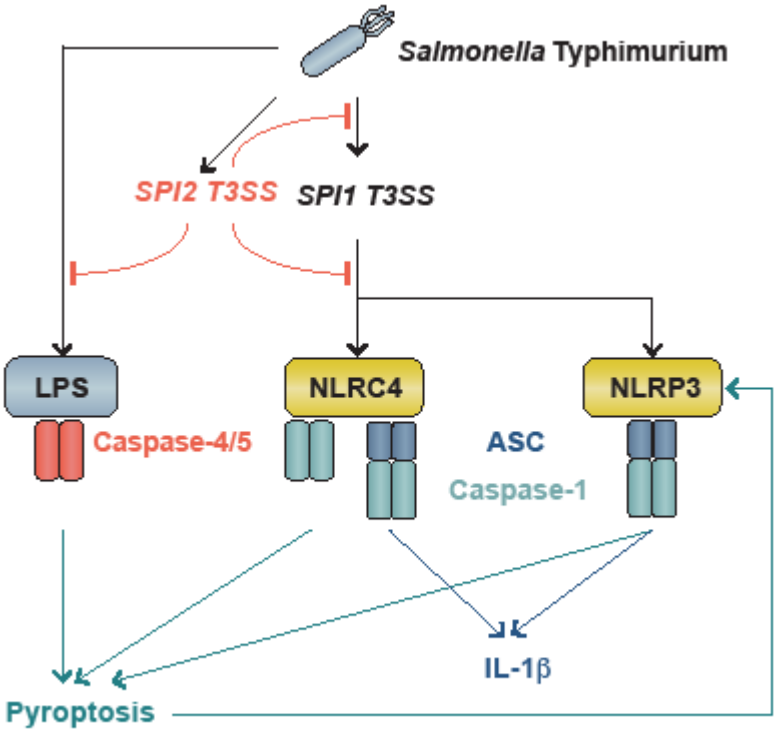
Summary sentence: The Gram-negative bacterium *Salmonella* Typhimurium suppresses human macrophage inflammasome responses via the *Salmonella* Pathogenicity Island 2



Running title: *Salmonella* Typhimurium subverts human inflammasomes

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Abbreviations

ASC, apoptosis-associated speck-like protein; BMM, bone-marrow derived macrophage; GSDMD, Gasdermin D; HMDM, human monocyte-derived macrophage; IL, interleukin; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; NAIP, NLR family, apoptosis inhibitory protein; NLRC4, NLR family CARD-domain containing protein 4; NLRP3, NLR family PYRIN-domain containing protein 3; SCV, *Salmonella*-containing vacuole; siRNA, small interfering RNA; SPI, *Salmonella* Pathogenicity Island; T3SS, type III secretion system.

Abstract

Inflammasomes are signaling hubs that activate inflammatory caspases to drive cytokine maturation and cell lysis. Inflammasome activation by *Salmonella* Typhimurium infection or *Salmonella*-derived molecules is extensively studied in murine myeloid cells. *Salmonella*-induced inflammasome signaling in human innate immune cells, is however, poorly characterized. Here, we show that *Salmonella* mutation to inactivate the *Salmonella* pathogenicity island-2 type III secretion system (SPI2 T3SS) potentiates *S.* Typhimurium-induced inflammasome responses from primary human macrophages, resulting in strong IL-1β production and macrophage death. Inactivation of the SPI1 T3SS diminished human macrophage responses to WT and ΔSPI2 *Salmonella*. *Salmonella* ΔSPI2 elicited a mixed inflammasome response from human myeloid cells, in which NLRC4 and NLRP3 perform somewhat redundant functions in generating IL-1β and inducing pyroptosis. Our data suggest that *Salmonella* employs the SPI2 T3SS to subvert SPI1-induced NLRP3 and NLRC4 inflammasome responses in human primary macrophages, in a species-specific immune evasion mechanism.

1 Introduction

The bacterium *Salmonella* can cause gastroenteritis or systemic infection in humans and mice [1]. Murine infection models have revealed the importance of the inflammasome signaling pathway in host protection against this bacterium [2, 3], particularly in myeloid innate immune cells (e.g. macrophages) and barrier defense cells (e.g. gut epithelial cells). Inflammasomes are signaling platforms that enable the activation of inflammatory caspases (caspase-1/4/5 in humans, caspase-1/11 in mouse) [4]. Upon activation, these cysteine proteases orchestrate a form of lytic cell death, termed pyroptosis [5]. Pyroptosis is mediated by Gasdermin D (GSDMD), which is cleaved by caspase-1/4/5 to generate GSDMD-p30 that forms plasma membrane pores to mediate osmotic swelling and cell lysis [6-9]. Caspase-1 also cleaves pro-inflammatory cytokines, such as IL-1β and IL-18, into their active, secreted forms.

Salmonella encodes virulence factors organized into *Salmonella* Pathogenicity Islands (SPIs). Of these, SPI1 [10] and SPI2 [11] have especially important functions during infection, and both encode type III secretion systems (T3SSs) that allow the injection of effector proteins into the host cell cytosol to modulate cell responses. Under *in vitro* growth conditions, SPI1 is strongly expressed during *Salmonella* log-growth phase, while SPI2 expression is upregulated during stationary-growth phase [11]. SPI2 virulence factors allow the establishment of the replication niche, the *Salmonella*-containing vacuole (SCV). Previous studies have demonstrated that multiple inflammasome sensors recognize *Salmonella* Typhimurium in murine cells. For example, flagellin [12] and components of the *Salmonella* SPI1 T3SS (e.g. prgI, prgJ) are recognized by murine NAIPs [13, 14] triggering the assembly of the NAIP/NLRC4 inflammasome which activates caspase-1 [15]. SCV lysis releases the

bacterium into the cytosol, where it is detected by the non-canonical inflammasome [16-18]. This inflammasome assembles when murine caspase-11, or human caspase-4/5, directly detect *Salmonella* lipopolysaccharide (LPS) [17]. Like caspase-1, these caspases cleave GSDMD to induce membrane pore formation and pyroptosis [6, 19]. Caspase-4/5/11 do not directly cleave IL-1β, but do so indirectly, as K^+ efflux via GSDMD pores activates the NLRP3 inflammasome, triggering caspase-1-dependent cytokine maturation [16, 20-22].

Despite their relevance for human infectious diseases, human host defense mechanisms (e.g. inflammasome responses) against *Salmonella* are poorly understood [23]. Here, we characterize the response of human macrophages to *Salmonella* Typhimurium infection, in which NLRP3 and NLRC4 inflammasomes function somewhat redundantly to drive cell death and IL-1β production. These studies highlight a new inflammasome subversion function for SPI2 specifically in human macrophages, and suggest a close interplay between SPI1 and SPI2 during *Salmonella* Typhimurium infection.

2 Materials and Methods

2.1 Macrophage differentiation and culture

Human CD14+ monocytes were isolated from screened buffy coats (provided by the Australian Red Cross) by density centrifugation with Ficoll-Paque Plus (GE Healthcare) followed by MACS-based CD14+ selection (Miltenyi Biotec). C57BL/6 mice were housed under specific pathogen-free conditions at the University of Queensland, and used as a source of bone marrow progenitors for differentiation into macrophages. Macrophage differentiation from human CD14+ monocytes and murine bone marrow was described previously [24]. Gene knockdown was performed using 500nM

Stealth siRNA, transfected into cells using Lipofectamine LTX (0.4%) and PLUS reagent (0.1%), and then incubated for 48 h. siRNA against ASC (HSS147064 and HSS147066; Life Technologies) versus a control siRNA (Medium GC Content Duplex #3; Life Technologies) were used, relative to a mock transfection control (transfection reagent only). All experiments with human primary cells and procedures involving mice were approved by the University of Queensland Medical Research Ethics Committee or the University of Queensland Animal Ethics Committee.

Primary macrophages and the THP-1 myeloid cell line were cultured in RPMI medium (Life Technologies) supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine with 5% CO2 at 37°C. Primary macrophages were additionally supplemented with 150 ng/mL endotoxin-free colony-stimulating factor (CSF)-1 (University of Queensland Protein Expression Facility). THP-1 cells were differentiated for 48 h with 10 ng/mL PMA (Sigma) prior to stimulation. Gene-deficient THP-1 cell lines were generated by CRISPR/Cas9 gene editing and were extensively validated before use [20, 25].

2.2 Bacterial culture, infection and gene expression analysis

Isogenic mutants of *Salmonella enterica* serovar Typhimurium SL1344, in which the SPI1 or SPI2 T3SS were rendered non-functional by targeting *invA* (ΔSPI1) or *ssaR* (ΔSPI2), were generated by gene replacement using the λ Red Recombinase system, where the chromosomal copy of the gene of interest was replaced with a kanamycin resistance cassette [26]. The strain in which both SPI1 and SPI2 T3SSs were disabled, by targeting *orgA* and *ssa*V (ΔSPI1/ΔSPI2), was previously described [27]. Stationary-phase bacteria were prepared by overnight culture in Luria broth (LB) at 37°C with agitation (200 rpm), supplemented with kanamycin (mutant strains only). Log-phase bacteria were prepared by sub-culture of 1:40 diluted stationary-phase bacteria to mid-log-phase in fresh LB (~3 h).

Bacteria were washed twice with sterile PBS before cell infection at a multiplicity of infection (MOI) of 25 bacteria/cell unless otherwise indicated. Following the addition of bacteria, cells were centrifuged at 500 *g* for 5 mins and incubated at 37°C in 5% CO2. 1 h post-infection, 20 μg/mL gentamicin was added to the cell culture media to kill extracellular bacteria. For analysis of bacterial gene expression, strains were cultured to log growth phase, subject to enzymatic lysis, and DNA-free total RNA was extracted by in-column purification (RNeasy mini kit, Qiagen). 1 μg of the purified RNA was transcribed to cDNA by incubation with random hexamers at 65°C for 5 mins (Invitrogen), before addition of SuperScript III reverse transcriptase and 0.1 M dithiothreitol (Invitrogen), and incubation at 25°C for 10 mins, 50°C for 50 mins, and 85°C for 5 mins. Relative cDNA abundance was quantified relative to the bacterial housekeeping gene *ftsZ* by SYBRGreen (Applied Biosystems) quantitative PCR using gene-specific primers.

2.3 Inflammasome assays

Primary macrophages were primed for 4 h with 100 ng/mL ultrapure *E. coli* K12 LPS (Invivogen) before replacing media with Opti-MEM containing 150 ng/mL CSF-1 plus an inflammasome agonist. Inflammasomes were triggered with *Salmonella* Typhimurium SL1344, or 10 μM nigericin (Sigma). Where indicated, cells were exposed to the caspase-1/4 inhibitor, VX765 (25 μM HMDM, 50 μM THP-1) or the NLRP3 inhibitor, MCC950 (10 μM), during the final 30 mins of cell priming, and maintained during cell stimulation. Triton fractionation to isolate the ASC speck without crosslinking was performed as previously reported [28], with the minor modification that centrifugation was performed at room temperature. Lactate dehydrogenase (LDH) release was monitored to measure cell death 4 h after inflammasome stimulation using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega), unless otherwise specified. IL-1β secretion into the culture medium was quantified 20 h after inflammasome stimulation by ELISA (IL-1β Ready-Set-Go! kits, eBioscience).

2.4 Immunoblotting

Immunoblot analysis was performed according to standard protocols [29]. Primary antibodies were rabbit anti-caspase-1 (Santa Cruz A-19, 1:1000), mouse anti-caspase-4 (Santa Cruz 4B9, 1:500), rabbit anti-caspase-5 (Abcam EP876Y, 1:1000), rabbit anti-IL-1β (Santa Cruz H-153, 1:1000), rabbit anti-ASC (Santa Cruz N15, 1:1000), and mouse anti-tubulin (Sigma-Aldrich B-5-1-2, 1:5000). Secondary antibodies were horse anti-mouse-HRP (Cell Signaling 7076, 1:5000), goat anti-rabbit-HRP (Cell Signaling 7074, 1:5000) and donkey anti-mouse-HRP (Cell Signaling 7076, 1:5000).

2.5 Statistical analysis

Data were analyzed for significant differences using a paired parametric Students t-test (single time point data) or a 2-way ANOVA (time course data) using Prism 7 (Graphpad). Differences were considered significant when the p-value was less than or equal to 0.05 (*), 0.01 (**), 0.001 (***), 0.0001 (****).

3 Results

3.1 SPI2 subverts human macrophage cell death and IL-1β responses

To characterize the inflammasome response of human macrophages against *Salmonella*, human monocyte-derived macrophages (HMDM) were infected with WT *Salmonella* Typhimurium SL1344 grown under SPI1-promoting conditions (log-phase) to mimic the early phase of host infection. HMDM were also infected with isogenic mutants of SL1344, in which SPI1 or SPI2 T3SS were rendered non-functional via deletion of key components of these systems (ΔSPI1, ΔSPI2).

Macrophages were spin-infected to promote *Salmonella* uptake by phagocytosis, and inflammasome responses such as cell death and cytokine production were monitored. WT *Salmonella* induced moderate macrophage death and IL-1β secretion, and this was markedly reduced by SPI1 mutation (**Fig. 1A-B**), confirming the importance of SPI1 in *Salmonella* recognition when this bacterium is grown under log-phase growth conditions. Unexpectedly, ΔSPI2 *Salmonella* induced stronger cell death (**Fig. 1A**) and IL-1β (**Fig. 1B**) responses than WT *Salmonella* in HMDM. ΔSPI2 *Salmonella*-induced macrophage death was rapid, evident as early as 1 h post-infection (**Supplementary Fig. 1**). Similar to bacteria grown to log phase, when *Salmonella* was grown to stationary growth phase, SPI2 mutation promoted HMDM cell death and IL-1β production, particularly at a high multiplicity of infection (**Supplementary Fig. 2**). Collectively, these data indicate that *Salmonella* SPI2 subverts the defense responses of human macrophages.

Murine macrophages infected with log-phase WT *Salmonella* are reported to mount strong inflammasome responses [30]. We confirmed this, by infecting murine bone marrow-derived macrophages (BMM) with log-phase *Salmonella* strains. *Salmonella* infection indeed elicited robust BMM cell death and IL-1β responses, via *Salmonella* SPI1 (**Fig. 1C-D**). Intriguingly, inactivation of the SPI2 T3SS did not affect *Salmonella*-induced cell death or IL-1β secretion in BMM, while SPI1 mutation strongly reduced such responses (**Fig. 1C-D**). This indicates that the SPI2 system subverts cell death and IL-1β responses in primary human but not murine macrophages, suggestive of a species-specific inflammasome evasion mechanism.

3.2 Human macrophage death and IL-1β production is elicited by Salmonella SPI1 and suppressed by SPI2

As *Salmonella* deficiency in the SPI1 T3SS abrogated HMDM cell death and IL-1β secretion, this suggested that these responses require macrophages to detect bacterial proteins translocated through the SPI1 T3SS, or components of the SPI1 T3SS itself [13, 14]. To determine whether the SPI2 T3SS machinery allows *Salmonella* to evade inflammasome responses elicited by the SPI1 T3SS system, we infected HMDMs with WT, ΔSPI2, ΔSPI1 and ΔSPI1/ΔSPI2 *Salmonella*. The potent cell death responses elicited by WT or ΔSPI2 *Salmonella* required SPI1 function, as these were ablated by SPI1 single or double mutation (ΔSPI1, ΔSPI1/ΔSPI2; **Fig. 2A**). IL-1β secretion induced by WT and ΔSPI2 *Salmonella* was also markedly suppressed when the SPI1 T3SS was inactivated (ΔSPI1, ΔSPI1/ΔSPI2, **Fig. 2B**). As SPI1 is required for active invasion of macrophages, we confirmed that *Salmonella* mutants doubly deficient for SPI1 and SPI2 were effectively internalized by HMDM in our assays. Bacterial uptake by phagocytosis, and bacterial survival, were indeed unaffected by SPI1/SPI2 double deficiency (**Supplementary Fig. 3**). In all, these data suggest a model in which *Salmonella* SPI2 suppresses SPI1-induced human macrophage cell death and IL-1β production.

We next examined whether SPI2 suppression of SPI1-induced responses occurs through bacterial transcriptional regulation. We monitored bacterial expression of flagellin loci (*fljB* and *fliC*), whose gene products are translocated into the macrophage cytosol via the SPI1 T3SS, and inflammasome-activating components of the SPI1 T3SS itself. SPI2 mutation did not upregulate expression of *fliC* (**Supplementary Fig. 4A**), but did promote expression of *fljB* and the SPI1 T3SS components, *prgI* and *prgJ* (**Supplementary Fig. 4B-D**). Conversely, SPI2-encoded genes, *ssaR* and *ssrB* were upregulated in strains with single or double SPI1 mutation (**Supplementary Fig. 4E-F**). This suggests that SPI2-dependent evasion of *Salmonella*-induced human macrophage responses may involve the

repression of inflammasome-activating components of the SPI1 T3SS and/or FljB. Importantly, SPI2-dependent inflammasome evasion is evident in human but not mouse macrophages; this suggests that SPI1-induced macrophage responses occur at distinct thresholds between species, and SPI2-driven mechanisms are only effective in evading the human response.

3.3 WT and ΔSPI2 Salmonella induces human myeloid cell death and IL-1β secretion via the inflammasome pathway

Macrophage cell death and IL-1β production are hallmarks of inflammasome signaling. To confirm that these responses induced by *Salmonella* ΔSPI2 were indeed mediated via the inflammasome pathway, we pre-treated HMDM with VX765, a caspase-1/4 inhibitor that blocks signaling by canonical inflammasomes and the caspase-4 non-canonical inflammasome [31]. VX765 blocked HMDM cell death (**Fig. 3A**) and IL-1β secretion (**Fig. 3B**) induced by WT and ΔSPI2 *Salmonella*. The impact of caspase-1/4 inhibition by VX765 was also assessed in infected THP-1 myeloid cells differentiated with PMA to resemble human macrophages. VX765 suppressed, but did not entirely block, THP-1 cell death induced by WT and ΔSPI2 *Salmonella* (**Fig. 3C**), indicating that other pathways (e.g. caspase-5) also contribute to death of ΔSPI2-infected THP-1, as we previously observed for THP-1 infected with WT *Salmonella* [20]. VX765 ablated IL-1β production induced by WT and ΔSPI2 *Salmonella* in THP-1 myeloid cells (**Fig. 3D**). WT and ΔSPI2 *Salmonella* thus elicit IL-1β and macrophage death via one or more inflammasome signaling pathways.

3.4 The NLRP3 inflammasome is redundant for HMDM responses to ΔSPI2 Salmonella

Salmonella and other Gram-negative bacteria can activate the non-canonical inflammasome during infection of murine and human macrophages [20-22, 32, 33]. Caspase-11/4/5 signaling in this

pathway leads to cell death and resultant activation of the NLRP3 inflammasome, thereby generating caspase-1-dependent IL-1β. The NLRP3 inflammasome is thus required for IL-1β production but not pyroptosis in these settings [20-22, 32, 33]. To determine whether log-phase ΔSPI2 *Salmonella* induces a similar pathway of non-canonical NLRP3 signaling in human primary macrophages, we treated LPS-primed HMDM with MCC950, a potent NLRP3 inhibitor [34], prior to infection with WT or ΔSPI2 *Salmonella*. HMDM were stimulated in parallel with the NLRP3 agonist, nigericin, as a positive control for NLRP3 activation and inhibition by MCC950 [34]. MCC950 indeed reduced HMDM death and IL-1β secretion in nigericin-stimulated macrophages (**Fig. 4A-B**). MCC950 did not, however, suppress pyroptosis or IL-1β production elicited by WT or ΔSPI2 *Salmonella*. As caspase activation leads to auto-processing, we also monitored the cleavage of caspase-1, caspase-4 and caspase-5 upon HMDM infection with WT versus ΔSPI2 *Salmonella*. WT and ΔSPI2 SL1344 triggered cleavage of caspase-1 to generate the 10 kDa free CARD domain, recently characterized to occur when active caspase-1 auto-processes to inactivate its protease function [28]. WT and ΔSPI2 SL1344 also triggered cleavage of caspase-1 to generate p33 caspase-1 that is part of an active caspase-1 p33/p10 species [28]. WT and ΔSPI2 SL1344 additionally induced cleavage of caspase-4, but not caspase-5, in HMDM (**Fig. 4C** and **Supplementary Fig. 5**). The caspase-1 p33 and caspase-4 p32 cleavage products were more intense in HMDM infected with ΔSPI2 as compared to WT SL1344 (**Fig. 4C**), suggesting that SPI2 mutation potentiates *Salmonella*-induced caspase-1/4 activation in HMDM. Cleavage of caspase-1 and IL-1β was unaffected by HMDM pre-treatment with MCC950 (**Fig. 4C**). Thus, the NLRP3 inflammasome is either dispensable for HMDM pyroptosis and IL-1β production during *Salmonella* infection, or its functions are redundant with another inflammasome. The caspase-4 non-canonical inflammasome likely contributes to infection-induced death of HMDM.

3.5 ASC is dispensable for Salmonella-induced pyroptosis but is required for maximal IL-1β production

ASC is a signal adaptor protein that is recruited to canonical inflammasomes, whereupon ASC polymerizes into a large, 'speck'-like complex. ASC specks are also formed during non-canonical inflammasome signaling, because GSDMD pores induced by the non-canonical caspase-4 inflammasome trigger a second wave of inflammasome signaling via NLRP3 [16, 20-22]. Our previous data suggested that the NLRP3 inflammasome activated by the non-canonical pathway was dispensable for IL-1β production during WT and ΔSPI2 *Salmonella* infection (**Fig. 4**). To confirm that a canonical inflammasome contributes to signaling in WT and ΔSPI2 *Salmonella*-infected cells, we monitored ASC speck formation using a biochemical assay in which cells are fractionated to isolate the ASC speck within the triton-insoluble fraction. THP-1 cells that were deficient for both *CASP4/5* and thus unable to signal via the non-canonical pathway were used for these studies, to identify canonical inflammasome responses without the potential for caspase-4/5-dependent NLRP3-induced ASC speck formation. Cells were also exposed to VX765 to prevent cell death and release of the ASC speck into the culture supernatant. ASC speck isolation in Δ*CASP4/5* THP-1 revealed that ΔSPI2 *Salmonella* induced strong ASC polymerization independently of the non-canonical pathway (**Fig. 5A**). Probing for caspase-1 further revealed the presence of active caspase-1 upon the ASC speck in ΔSPI2-infected THP-1 (p33 band, **Fig. 5A**). This confirmed that ΔSPI2 *Salmonella* elicits signaling by a canonical ASC-caspase-1 inflammasome.

ASC is required by canonical inflammasomes for caspase-1-dependent IL-1β production. To determine whether *Salmonella*-induced HMDM inflammasome responses required ASC, we silenced *ASC* expression using siRNA, relative to controls (mock transfection and non-targeting siRNA, **Fig. 5B**). 48 h later, HMDMs were infected with *Salmonella* strains, or exposed to nigericin to activate

NLRP3. ASC silencing suppressed nigericin-induced cell death and IL-1β production (**Fig. 5C-D**), indicating that the level of ASC knockdown (**Fig. 5B**) was sufficient for functional ablation. ASC knockdown did not affect HMDM death induced by WT or ΔSPI2 *Salmonella* (**Fig. 5C**). ASC knockdown did, however, significantly reduce IL-1β production from HMDM infected with WT or ΔSPI2 *Salmonella* (**Fig. 5D**). In all, these data suggest that polymeric ASC, engaged through a canonical inflammasome pathway, contributes to IL-1β production but is dispensable for pyroptosis induced by WT or ΔSPI2 *Salmonella*. Such a profile is consistent with signaling by NLRC4, in which ASC is required for IL-1β production but is not absolutely required for macrophage death [30, 35].

3.6 The NLRC4 and NLRP3 inflammasomes function redundantly to induce IL-1β production and pyroptosis during human myeloid cell infection with ΔSPI2

The NLRC4 co-receptor, NAIP, senses various *Salmonella* proteins (e.g. prgJ and prgI, flagellin) and the NAIP/NLRC4 inflammasome is thus a strong candidate for inflammasome responses to *Salmonella* in humans [23]. We next sought to determine whether NLRC4 is the canonical inflammasome responsible for IL-1β production and pyroptosis elicited by WT and ΔSPI2 *Salmonella*. THP-1 cells sufficient or deficient for *NLRC4* (Cas9 versus *ΔNLRC4*) were primed with LPS, infected with WT and ΔSPI2 *Salmonella*, and monitored for cell death and IL-1β secretion. Previous studies in *Salmonella*-infected murine macrophages showed functional redundancy between the NLRC4 and NLRP3 inflammasomes [27, 36], so we also inhibited NLRP3 function in these cells, by exposing them to MCC950 prior to infection. Alternatively, cells were pre-treated with VX765 to block caspase-1/4-dependent responses. THP-1 death induced by WT *Salmonella* was suppressed by *NLRC4* deficiency or caspase-1/4 inhibition, and was unaffected by MCC950 (**Fig. 6A**), indicating that NLRC4-dependent caspase-1 drives pyroptosis in this context. Cell death induced by ΔSPI2 *Salmonella* was suppressed by MCC950 only when NLRC4 function was additionally ablated,

indicating that the NLRC4 and NLRP3 inflammasomes perform redundant functions in ΔSPI2-induced pyroptosis (**Fig. 6A**). Of note, THP-1 death induced by WT and ΔSPI2 *Salmonella* was only partially suppressed by functional ablation of NLRP3 and NLRC4 (**Fig. 6A**), or inhibition of caspase-1/4 (**Figs. 3C, 6A**), indicating that other pathways also contribute to death in this cell type; this is consistent with a role for the caspase-5 non-canonical inflammasome in driving THP-1 pyroptosis as we have previously demonstrated during WT *Salmonella* infection of these cells [20]. By comparison, caspase-1/4 inhibition ablated HMDM death induced by WT and ΔSPI2 *Salmonella* infection (**Fig. 3A**). IL-1β production elicited by THP-1 infection with WT and ΔSPI2 *Salmonella* showed a similar profile to THP-1 cell death responses to infection, in which *NLRC4* deficiency sensitized cells to inhibition by MCC950 (**Fig. 6B**), and NLRP3 inhibition ablated the residual IL-1β response of *NLRC4*-deficient cells. Together, these data indicate that ΔSPI2 *Salmonella* induces pyroptosis and IL-1β production via the redundant activities of the NLRC4 and NLRP3 inflammasomes.

4 Discussion

The inflammasome response to *Salmonella* Typhimurium has been characterized in detail in murine macrophages. Murine macrophage infection with stationary-phase *Salmonella* Typhimurium initially leads to *Salmonella* sequestration within the SCV, but host cell mechanisms eventually rupture the SCV to release the bacterium into the macrophage cytosol [37]. *Salmonella* LPS thereby becomes exposed to caspase-11, activating non-canonical inflammasome signaling and inducing the pyroptotic death of the host cell [37]. Caspase-11-induced death also induces NLRP3-dependent IL-1β production [16, 17, 21, 27, 37]. By contrast, murine macrophages infected with log-phase *S.* Typhimurium rapidly sense this bacterium via the NLRC4 inflammasome, leading to caspase-1-dependent pyroptosis and secretion of mature IL-1β [38]. Herein, NAIPs sense the *Salmonella* SPI1 T3SS and bacterial proteins translocated through this apparatus, triggering NLRC4 inflammasome

activation and signaling [15]. Log-phase *Salmonella* also activates signaling by the NLRP3 inflammasome in murine macrophages, which provides some functional redundancy with the NLRC4 response [36].

Human innate immune responses to *Salmonella* are less well characterized [23] but our study suggests the dual NLRP3/NLRC4 inflammasome responses of mouse macrophages to this bacterium [27, 36] are also conserved in human macrophages. Our data indicate that during human myeloid cell infection with log-phase WT and ΔSPI2 *S.* Typhimurium, both the NLRP3 and NLRC4 inflammasomes pathways are engaged, leading to production of mature IL-1β and cell death. Co-signaling by these canonical inflammasomes may be a mechanism for human myeloid cells to ensure host defense against intracellular *Salmonella*. Whilst functional co-deficiency of NLRP3 and NLRC4 ablated IL-1β responses, it suppressed but did not ablate the cell death response of THP-1 infected with WT and ΔSPI2 *S.* Typhimurium. The caspase-1/4 inhibitor VX765 suppressed THP-1 death to a similar extent as functional co-deficiency of NLRP3 and NLRC4. This suggests that alternative pathways contribute to the infection-induced cell death response of THP-1; such pathways may include pyroptosis induced by the caspase-5 non-canonical inflammasome [20], or perhaps inflammasome/caspase8-induced apoptotic pathways that are enabled upon caspase-1/4 inhibition [39-41]. In HMDM, however, VX765 pre-treatment ablated infection-induced cell death, suggesting that caspase-1/4 is the sole driver of this response in primary cells. Human-specific inflammasome regulators (e.g. COPs and POPs) [42] may also modulate human inflammasome responses to infection.

The human macrophage inflammasome response to WT *Salmonella* was promoted by inactivation of the SPI2 T3SS, and macrophage inflammasome responses to WT or ΔSPI2 *Salmonella* were ablated upon SPI1 T3SS inactivation. This suggests a model in which the *Salmonella* SPI1 system is a

primary driver of human macrophage inflammasome responses, and the SPI2 T3SS enables bacterial evasion of this response. Strong expression of SPI1 or SPI2 requires different environmental cues, as SPI1-encoded virulence factors are highly expressed during the invasion stage of infection, while SPI2 virulence factors are upregulated within the intra-macrophage environment [43, 44]. SPI2 functions are critical for the establishment and maintenance of the SCV, and are proposed to be essential for bacterial proliferation *in cellulo* [44, 45] and *in vivo,* in mice [46] and other mammals [47, 48]. Interestingly, SPI2 mutation promoted human macrophage inflammasome responses to both log and stationary phase *Salmonella*, suggesting an unexpected role for SPI2 effectors during macrophage infection under log-phase growth conditions. This contrasts with murine macrophage infection under stationary-phase growth conditions, where SPI2 function was required for strong inflammasome responses in unprimed cells [27]. Such divergent effects of SPI2 mutation may reflect species-specific macrophage responses, or differences caused by macrophage priming. Given that SPI2 effectors such as SifA maintain SCV integrity, it is possible that SPI2 mutations may destabilize the SCV and thereby increase delivery of *Salmonella* to the cytoplasm for sensing by cytosolic pattern-recognition receptors. However, inflammasome responses to ΔSPI2 *Salmonella* were rapid, occurring within 1 h of human macrophage infection, whereas SCV destabilization in Δ*sifA*-infected murine macrophages occurs much later, around 8 h post-infection [49]. The rapidity of the human macrophage response to ΔSPI2 *Salmonella* suggests that SPI2 subverts inflammasome responses independently of its characterized functions in establishing and maintaining the SCV. Indeed, SPI2 effectors control a wide variety of other processes that may allow *Salmonella* to evade inflammasomes in human macrophages [11]. In allowing *Salmonella* to subvert SPI1-induced pyroptosis, SPI2 may support the SPI1-driven active infection of human macrophages, and intra-macrophage bacterial survival. In keeping with this, we observed that some NLRC4-activating bacterial proteins were more strongly expressed at the mRNA level in SPI2 mutant *Salmonella*, suggesting that SPI2-mediated suppression of *fljB*, *prgI* and *prgJ* expression may be one mechanism by which SPI2 enables evasion of human inflammasome responses. Future research should clarify the

mechanism by which the SPI2 T3SS subverts human macrophage inflammasome responses, and the importance of such a mechanism for infection of other cell types (e.g. epithelial cells) and by other *Salmonella* strains (e.g. *Salmonella* Typhi).

This study provides insight into the virulence mechanisms employed by bacteria to evade human innate immune defenses. Our data suggests new functions for SPI2 virulence factors in the subversion of inflammasome responses specifically in human macrophages, and reveals an unexpected interplay between the SPI1 and SPI2 systems of *Salmonella* Typhimurium.

5 Acknowledgements

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6 Authorship

D. Bierschenk, M.M., and D. Boucher designed, executed and analyzed experiments. M.M. generated the *Salmonella* single mutants used in this study. F.M., P.J.B. and S.L.M. generated the gene-deficient THP-1 lines used in this study. D. Boucher and K. Schroder designed the project and supervised the study. D. Bierschenk, D. Boucher, and K.S. wrote the paper with input from all authors.

7 Disclosure

A/Prof Schroder is a co-inventor on patent applications for NLRP3 inhibitors which have been licensed to Inflazome Ltd, a company headquartered in Dublin, Ireland. Inflazome is developing drugs that target the NLRP3 inflammasome to address unmet clinical needs in inflammatory disease. The authors have no further conflicts of interest to declare.

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Figures

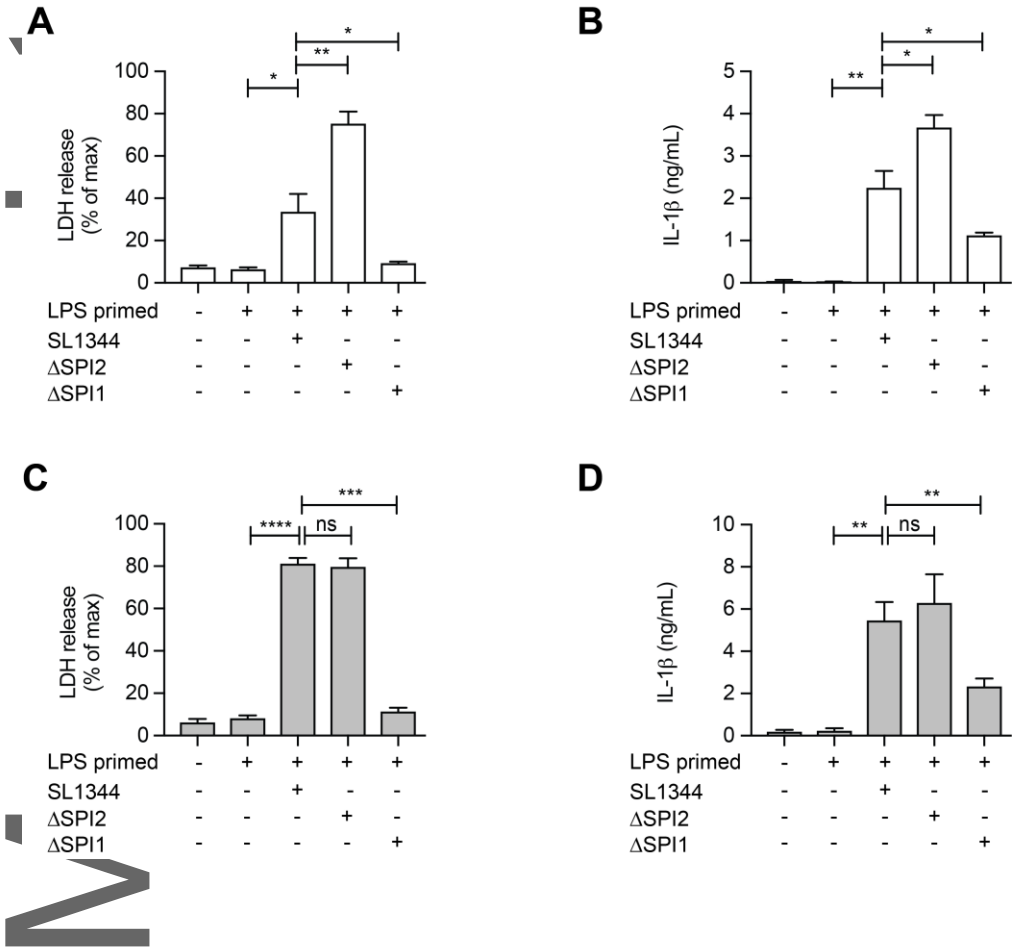

Figure 1. *S.* Typhimurium SPI2 subverts inflammasome pathways in human macrophages. HMDM **(A, B)** and BMM **(C, D)** were treated with 100 ng/ml LPS for 4 h prior to *S.* Typhimurium infection (MOI 25, log-phase). Cell death was assayed by LDH release 4 h post-infection, while IL-1β secretion was measured by ELISA 20 h post-infection. Data are mean + SEM of data pooled from five independent human donors **(A, B)** or four independent experiments **(C, D)**.

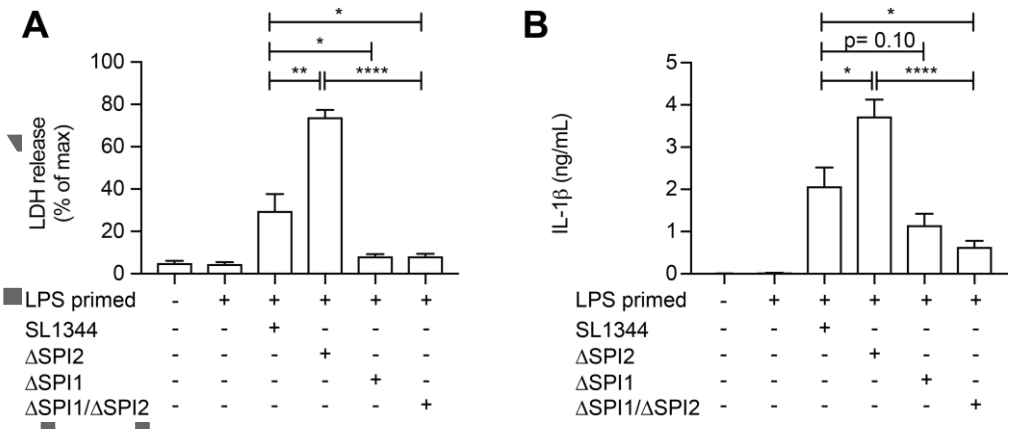

Figure 2. ΔSPI2-induced HMDM responses require SPI1 function. (A, B) HMDMs were primed with 100 ng/ml LPS for 4 h prior to *S.* Typhimurium infection (MOI 25, log-phase). **(A)** LDH release and **(B)** IL-1β secretion were measured at 4 h or 20 h post-infection, respectively. All data are mean + SEM of data pooled from seven independent human donors.

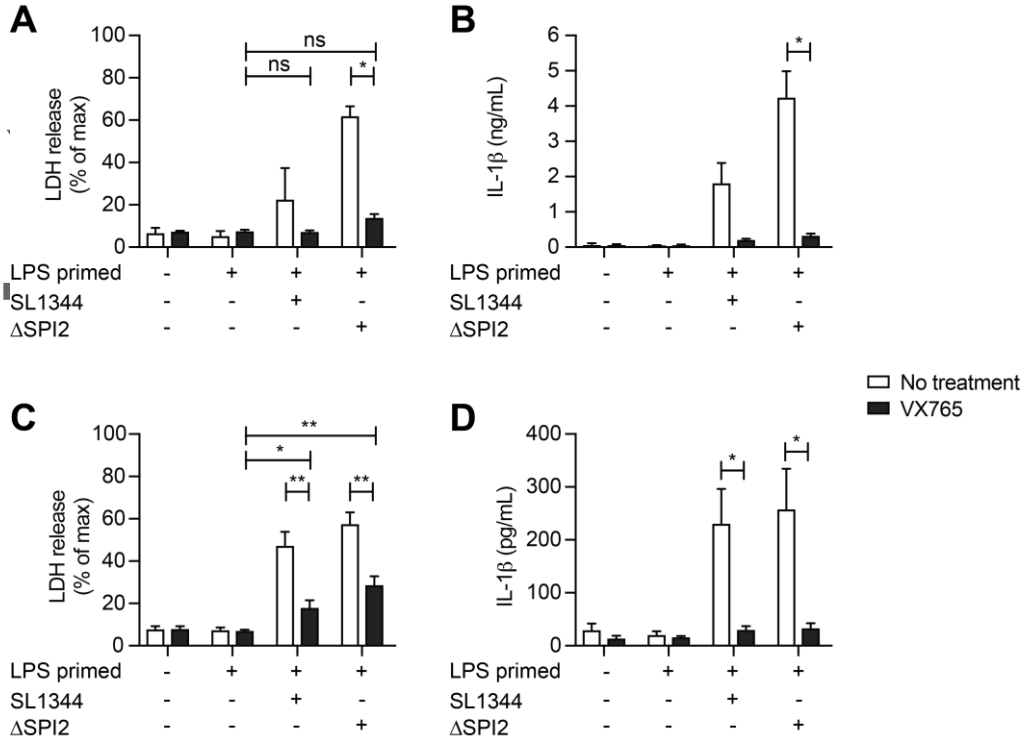

Figure 3. Cell death and IL-1β production upon ΔSPI2 *S.* Typhimurium infection requires caspase-1/4 and hence inflammasome signaling. HMDM (**A, B**) or PMA-differentiated THP-1 cells (**C, D**) were primed with 100 ng/ml LPS for 4 h and then infected with *S.* Typhimurium (MOI 25, log-phase). Caspase-1/4 activity was blocked by cell exposure to VX765 in the final 30 mins of cell priming. (**A, C**) LDH release was measured after 4 h, and (**B, D**) IL-1β secretion was measured after 20 h. Data are mean + SEM of data pooled from three **(A, B)** or five **(C, D)** independent experiments.

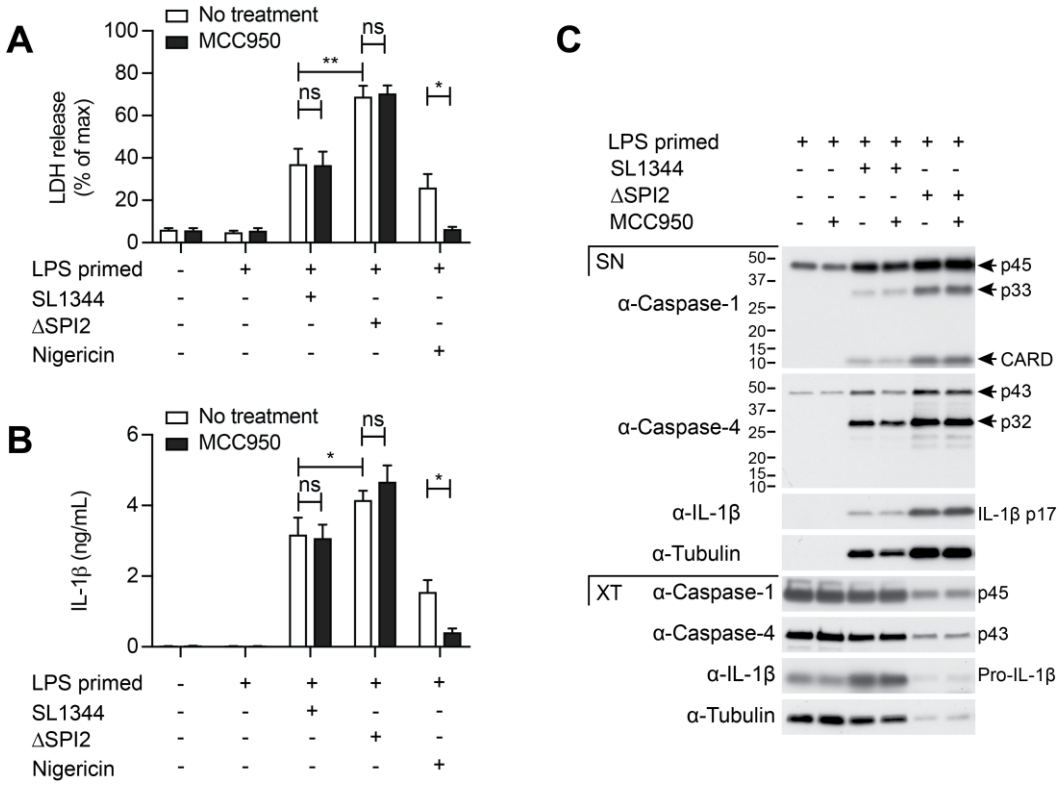

Figure 4. NLRP3 is dispensable or redundant for the HMDM response to WT and ΔSPI2 *S.* Typhimurium. HMDM were primed with 100 ng/ml LPS for 4 h prior to *S.* Typhimurium infection (MOI 25, log-phase) or 10 μM nigericin treatment. NLRP3 activity was suppressed by exposing HMDM to 10 μM MCC950 in the final 30 mins of priming. Cells were then analyzed for **(A)** LDH release 4 h post-infection, **(B)** IL-1β secretion 20 h post-infection, or **(C)** caspase-1, caspase-4 and pro-IL-1β cleavage 4 h post-infection. Graphs are mean + SEM of data pooled from eight **(A, B)** independent donors, while immunoblots are representative of three independent experiments.

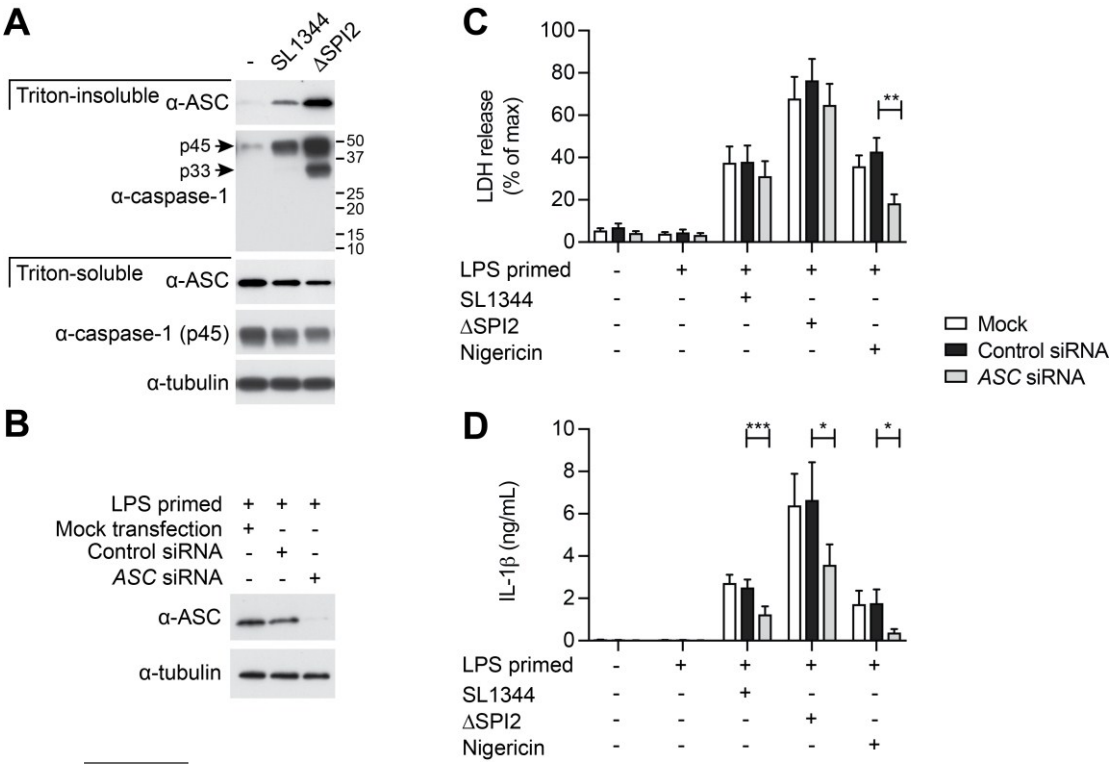

Figure 5. A canonical inflammasome pathway drives ASC speck formation, which is required for WT and ΔSPI2-induced HMDM IL-1β but is dispensable for pyroptosis. (**A**) PMA-differentiated *CASP4/5*-deficient THP-1 cells were primed with 100 ng/ml LPS for 4 h, and were then left uninfected or were infected with *S.* Typhimurium (MOI 25, log-phase), in the presence of 25 μM VX765 to suppress cell death and cellular release of the ASC speck. 4 h later, cells were then fractionated for polymeric ASC (Triton X-100 insoluble) or monomeric ASC (Triton X-100 soluble), and cell fractions were analysed by immunoblot. (**B-D**) HMDMs were mock transfected, or transfected with siRNA (control, *ASC*-targeting) for 48 h: **(B)** HMDMs were primed for 4 h with 100 ng/ml LPS before cell extracts were analysed for ASC knockdown by immunoblot. (**C-D**) HMDMs were primed for 4 h with 100 ng/ml LPS prior to *S.* Typhimurium infection (MOI 25, log-phase), or treatment with 10 μM nigericin. LDH release and IL-1β secretion were monitored at 4 h and 20 h post-infection, respectively. Immunoblots are representative of data from three independent experiments (**A, B**), while graphs show mean + SEM of data pooled from six to seven independent donors (**C, D**).

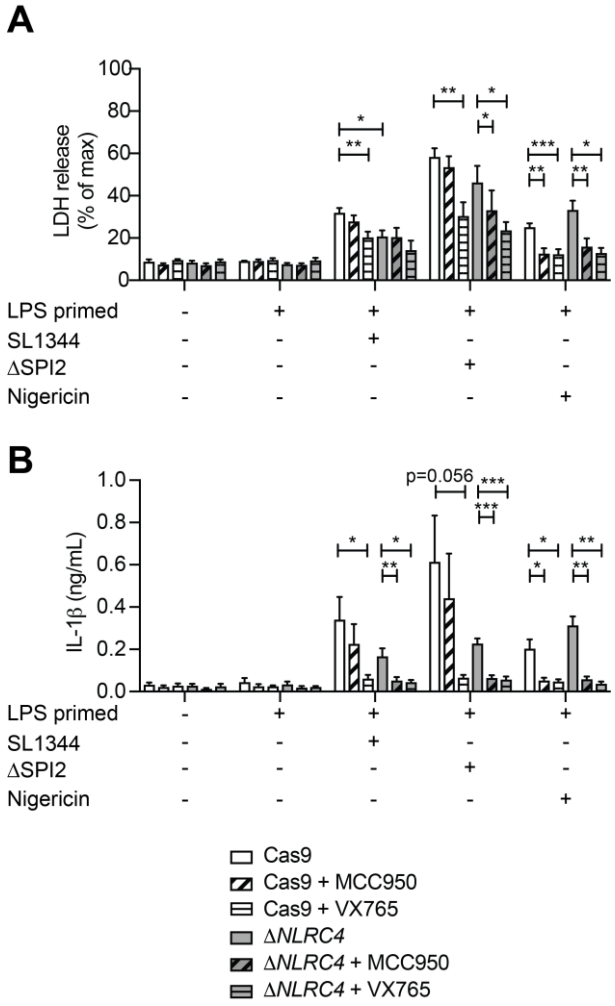

Figure 6. The NLRC4 and NLRP3 inflammasomes contribute to cell death, and drive IL-1β secretion during THP-1 infection with *S.* Typhimurium. PMA-differentiated THP-1 cells were primed with 100 ng/ml LPS for 4 h and then treated with 10 μM nigericin or infected with *S.* Typhimurium (MOI 25, log-phase). Cells were exposed to MCC950 or VX765 in the final 30 mins of priming. **(A)** LDH release was monitored after 4 h, and **(B)** IL-1β secretion was measured after 20 h. Data are mean + SEM of five independent experiments.