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The Neutrophil NLRC4 Inflammasome Selectively Promotes IL-1β Maturation without Pyroptosis during Acute *Salmonella* Challenge

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SUMMARY

The macrophage NLRC4 inflammasome drives potent innate immune responses against *Salmonella* by eliciting caspase-1-dependent proinflammatory cytokine production (e.g., interleukin-1β [IL-1β]) and pyroptotic cell death. However, the potential contribution of other cell types to inflammasome-mediated host defense against *Salmonella* was unclear. Here, we demonstrate that neutrophils, typically viewed as cellular targets of IL-1β, themselves activate the NLRC4 inflammasome during acute *Salmonella* infection and are a major cell compartment for IL-1β production during acute peritoneal challenge in vivo. Importantly, unlike macrophages, neutrophils do not undergo pyroptosis upon NLRC4 inflammasome activation. The resistance of neutrophils to pyroptotic death is unique among inflammasome-signaling cells so far described and allows neutrophils to sustain IL-1β production at a site of infection without compromising the crucial inflammasome-independent antimicrobial effector functions that would be lost if neutrophils rapidly lysed upon caspase-1 activation. Inflammasome pathway modification in neutrophils thus maximizes host proinflammatory and antimicrobial responses during pathogen challenge.

INTRODUCTION

The innate immune system engages an array of pattern-recognition receptors (PRRs) to detect signals of host infection, tissue injury, and cellular stress. Among these receptors are the Toll-like receptors, C-type lectins, cytoplasmic nucleic acid receptors (e.g., RIG-like helicases and HIN-200 proteins), and Nod-like receptors (NLRs). NLRs are activated by pathogen-associated molecules and host-derived alarmins indicating cellular injury or stress, and they are potent mediators of inflammation. The human NLR family has 22 members and is defined by the presence of a central NACHT domain that triggers self-oligomerization. A functional subgroup of the NLR family forms high-molecular-weight complexes known as inflammasomes (e.g., NLRP1, NLRP3, NLRP6, NLRP12, and NLRC4) (Schroder and Tschopp, 2010).

Inflammasomes are "danger" sensor complexes that trigger immune system activation, and most of the biological characterization of inflammasomes relies upon studies in macrophages and dendritic cells. Inflammasome activation involves the oligomerization of an NLR scaffold, followed by the recruitment, clustering, and autoactivation of a proinflammatory caspase, caspase-1, usually via a protein adaptor ASC (Schroder and Tschopp, 2010). Upon activation, caspase-1 mediates the maturation and secretion of proinflammatory cytokines such as interleukin-1β (IL-1β) and IL-18 and induces a rapid lytic form of inflammatory cell death (pyroptosis). These caspase-1 functions together coordinate host-protective inflammatory and antimicrobial responses. IL-1β and IL-18 drive proinflammatory responses, including the recruitment and activation of phagocytes (Chen and Schroder, 2013), while pyroptotic cell death releases the intracellular pathogens of macrophages into the extracellular environment, rendering them susceptible to neutrophil-mediated destruction (Miao et al., 2010a). Specific Gram-negative bacteria encoding type 3 or 4 secretion systems, such as *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium [*Stm*]) and *Pseudomonas aeruginosa* (*P. aeruginosa*), trigger activation of the NLRC4 inflammasome (Mariathasan et al., 2004; Miao et al., 2008). NLRC4 activation by *Stm* occurs upon cytosolic recognition of bacterial ligands (needle protein, PrgJ, flagellin) following their translocation into the host cell cytosol by the *Stm* pathogenicity island-1 type 3 secretion system (SPI1-T3SS) (Miao et al., 2010b; Rayamajhi et al., 2013; Yang et al., 2013). NLRC4 activation by these ligands is mediated by NAIP coreceptors, also encoded within the NLR family (Kofoed and Vance, 2011; Rayamajhi et al., 2013; Yang et al., 2013; Zhao et al., 2011). NLRC4 has a CARD domain and so can interact

directly with pro-caspase-1 through homotypic CARD-CARD interactions, bypassing an absolute requirement for the ASC inflammasome adaptor. This ASC-independent NLRC4 inflammasome was reported to form a death complex that drives caspase-1-dependent macrophage lysis (Broz et al., 2010b). However, efficient cytokine processing by the NLRC4 inflammasome in macrophages requires ASC (Broz et al., 2010b).

Although recent years have seen great progress in our understanding of the molecular mechanisms underlying NLRC4 inflammasome activation, several key questions regarding NLRC4 biology remain outstanding. One such question is the identity of the cell types responsible for initiating NLRC4 pathways during in vivo infection; most NLRC4 research to date has concentrated on macrophages and dendritic cells without clear data indicating these are the dominant cell types triggering NLRC4 activation, IL-1β/IL-18 production, and pyroptosis in vivo. Another key question is the extent to which inflammasome-dependent processes (cytokine production versus cell death) are interregulated. Production of active IL-1β/IL-18 is controlled by caspase-1 at two levels: cytokine processing from the inactive precursor to the mature form, and cytokine release from the cytosol to the extracellular space. It is currently unclear whether the release of mature cytokine occurs solely via an active caspase-1-dependent unconventional secretion pathway (Keller et al., 2008) or whether passive release during pyroptosis contributes to cytokine export. It is thus presently unresolved whether pyroptosis is actually necessary for full inflammasome-dependent cytokine production (through contributing to cytokine release) or, alternatively, whether pyroptosis serves to limit cytokine production by preventing the further processing of pro-caspase-1, pro-IL-1, and pro-IL-18 cytosolic pools. If the latter, this raises the further intriguing question of how inflammasome pathways trigger sustained cytokine production during in vivo infection and inflammatory disease, when inflammasome-activated cells would be expected to rapidly lyse, limiting their ability to produce further cytokines.

Neutrophils are the first cells recruited in large quantities to a site of infection or injury, and inflammasome activity itself induces IL-1-dependent neutrophil influx in vivo. The possibility that neutrophils themselves are competent for NLRC4 inflammasome function and thereby contribute to IL-1-dependent amplification of neutrophil recruitment/activation and pathogen clearance has not been carefully investigated. Herein, we report that the neutrophil NLRC4 inflammasome selectively promotes *Stm*-dependent cytokine processing, but not pyroptosis, in vitro and in vivo. This ability of neutrophils to produce caspase-1-dependent IL-1β without concomitant cell death is unique among NLRC4-signaling cells so far described and challenges the dogma that IL-1β production and pyroptosis are always initiated together. Our findings explain the extended kinetics of inflammasome-dependent IL-1β production during acute infection.

RESULTS

Neutrophils Express Multiple NLRs

Neutrophils express a wide variety of PRRs (Thomas and Schroder, 2013) and are likely candidates for NLR function. However, the expression and potential function of most NLRs in neutrophils is unclear (Thomas and Schroder, 2013). To date, only one recent study has specifically shown a function for a neutrophil inflammasome, that of NLRP3, in mediating host inflammatory responses during in vivo infection (Cho et al., 2012). In order to define the neutrophil NLR repertoire, we examined the mRNA expression of all 22 NLR family members among purified immune cell fractions from human blood. Human blood was separated into polymorphonuclear cell (PMN) and peripheral blood mononuclear cell (PBMC) fractions by discontinuous density sedimentation. The PMN and PBMC fractions were then further purified on the basis of cell-surface markers. A total of 15 of the 22 human NLRs are reliably detectable in such cells (Figure 1A). Human neutrophils purified from the PMN fraction by CD16-positive selection express numerous NLRs, including the inflammasome scaffolds *NLRP1*, *NLRP3*, *NLRP6*, *NLRP12*, and *NLRC4* (Figure 1A; Figure S1). For example, *NLRP3* and *NLRC4* mRNAs were expressed in purified human neutrophils at similar or greater levels than other cell types for which NLRP3 and NLRC4 function is well characterized, such as $CD14^+$ monocytes, monocyte-derived macrophages, and monocyte-derived dendritic cells (Figures 1B and 1C), and *NLRP12* mRNA showed a striking enrichment in neutrophils versus other white blood cell populations within the panel (Figure 1D). Mouse bone marrow neutrophils (BMNs) also expressed *Nlrp3*, *Nlrc4*, and *Nlrp1a* at similar or greater levels to bone marrow-derived macrophages (BMDMs) and/or bone marrow-derived dendritic cells (BMDCs) (Figures 1E and 1F; Figure S1). In human and murine macrophages, cell stimulation with the TLR4 agonist, lipopolysaccharide (LPS) upregulates the expression of *Nlrp3* and *Nlrc4* (Schroder et al., 2012b). Similarly, we observed LPS-dependent upregulation of *Nlrp3* and *Nlrc4* expression in murine neutrophils (Figure S2). Constitutive and inducible expression of inflammasome-forming NLRs in neutrophils suggests that like other PRRs, inflammasomes may modulate neutrophil responses during inflammation.

The Neutrophil NLRC4 Inflammasome Drives Caspase-1 and IL-1β Activation

Neutrophil expression of *NLRC4* mRNA suggested that neutrophils may participate in inflammasome-dependent antimicrobial responses against *Salmonella*. We investigated this possibility by infecting mature bone marrow neutrophils purified from wild-type (WT) and caspase-1/11-deficient ($Ice^{-/-}$) mice. Neutrophils were primed with LPS to induce pro-IL-1β expression, prior to infection with varying doses of log-phase *Stm* SL1344. BMDMs, which are competent for NLRC4-dependent pathways (Broz et al., 2010b; Miao et al., 2010b), were prepared and treated in parallel for comparison. Treatment with LPS alone did not stimulate IL-1β secretion in either neutrophils or macrophages, but 5 hr *Stm* infection triggered robust IL-1β secretion in LPS-primed neutrophils (Figure 2A), similar to infected WT macrophages (Figure 2B). *Stm*-induced IL-1β secretion in both neutrophils and macrophages was ablated in $Ice^{-/-}$ cells, indicating that IL-1β production was mediated by an inflammasome. Of note, the optimal dose of *Stm* and the kinetics of IL-1β secretion varied slightly between neutrophils and macrophages.

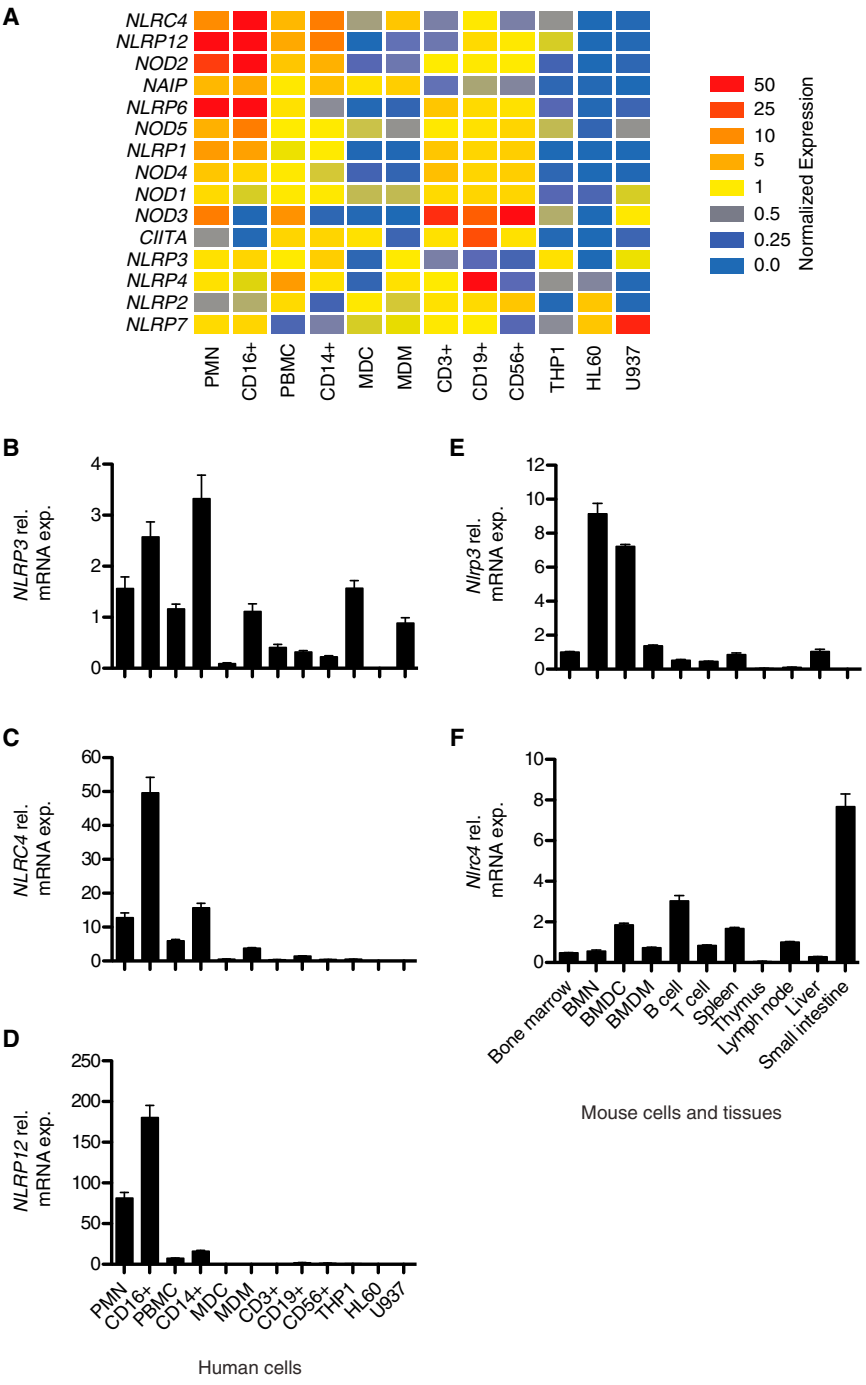


Figure 1. Human and Mouse Neutrophils Express Multiple NLRs

(A) Heatmap showing median-normalized quantitative PCR profiles of all NLRs reliably detected in human blood cell populations or myeloid cell lines.

(B–D) Expression profile detail for *NLRP3* (B), *NLRC4* (C), and *NLRP12* (D) in purified human leukocyte populations and cell lines.

(E and F) Neutrophil expression of *Nlrp3* (E) and *Nlrc4* (F) was confirmed in a panel of mouse tissues and purified cell populations.

Data are mean + SD of technical replicates and are representative of three independent experiments. Human cells: polymorphonuclear fraction (PMN; containing neutrophils and eosinophils), neutrophils ($CD16^+$ cells from the PMN fraction, which excludes monocytes), bulk peripheral blood mononuclear cell fraction (PBMC), monocytes ($CD14^+$ cells from the PBMC fraction), monocyte-derived dendritic cells (MDC), monocyte-derived macrophages (MDM), T cells ($CD3^+$), B cells ($CD19^+$), natural killer cells ($CD56^+$), and the monocytic cell lines THP-1, HL60, and U937. Mouse cells: bone marrow neutrophils (BMNs), bone marrow-derived dendritic cells (BMDCs), bone marrow-derived macrophages (BMDMs), and splenic B and T lymphocytes. See also Figures S1 and S2.

Efficient *Stm*-dependent cytokine production by the macrophage NLRC4 inflammasome requires the ASC inflammasome adaptor (Broz et al., 2010b). We found that ASC was also required for optimal *Stm*-dependent IL-1β production from neutrophils (Figure 2C), similar to macrophages (Figure 2D). At higher multiplicities of infection (MOIs), moderate ASC-independent IL-1β production was apparent for both neutrophils and macrophages, and we confirmed by immunoblotting that neutrophil IL-1β released in an ASC-independent manner was the mature form (Figure 2E). As *Stm*-dependent IL-1β production was maximal in neutrophils and macrophages at distinct multiplicities of infection, for subsequent in vitro experiments, we infected neutrophils and macrophages with *Stm* doses that yield robust IL-1β production (MOIs of 25 and 5, respectively).

In macrophages, IL-1β production during acute *Stm* infection relies upon the *Stm* SPI1-T3SS needle (Miao et al., 2010b), which translocates bacterial proteins into the macrophage cytosol for recognition by the NAIP coreceptors of the NLRC4 inflammasome (Kofoed and Vance, 2011; Rayamajhi et al., 2013; Yang et al., 2013; Zhao et al., 2011). NLRP3 also contributes to *Stm* sensing in unprimed macrophages at later time points of infection (Broz et al., 2010a; Man et al., 2014); the late time course of NLRP3 function in unprimed cells presumably reflects the functional requirement for infection-induced NLRP3 expression. We thus examined whether similar host and pathogen determinants underlie IL-1β production from neutrophils. We infected WT or inflammasome-deficient neutrophils and macrophages with *Stm* or its isogenic mutant (ΔSPI1) and assayed IL-1β production at 5 hr postinfection. The *Stm* SPI1-T3SS translocon was required for IL-1β secretion from both macrophages and neutrophils (Figure 2E; Figures S3A and S3B). As for macrophages, NLRC4 was essential for

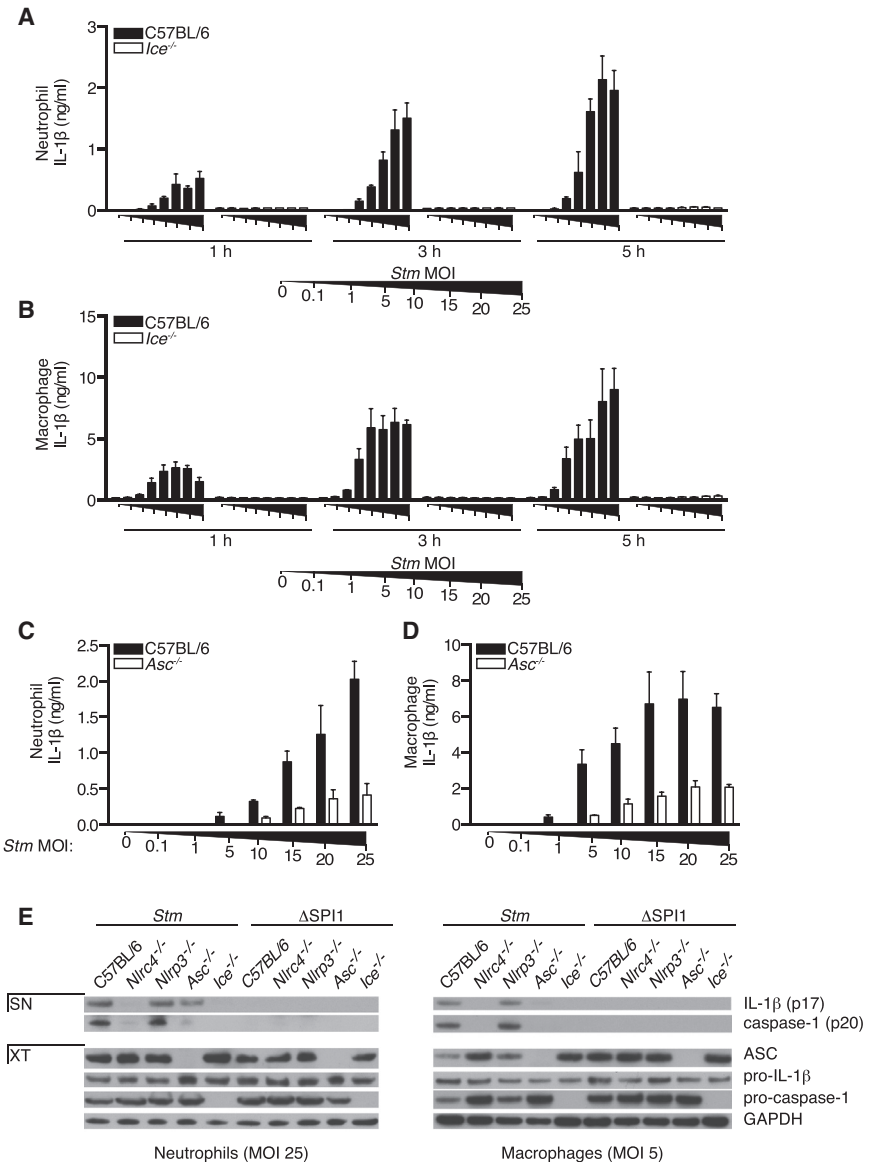


Figure 2. Neutrophil NLRC4 Activation Triggers Caspase-1 and IL-1β Cleavage and Secretion

(A–E) Bone marrow neutrophils (A, C, and E, left panel) or bone marrow-derived macrophages (B, D, and E, right panel) were primed with 100 ng/ml LPS for 4 hr before infection with *S.* Typhimurium (*Stm*). Cells were infected with increasing doses of *Stm*, and IL-1β secretion was measured by ELISA at 1, 3, and 5 hr postinfection (A and B) or 5 hr postinfection (C and D).

(E) Cells were infected with *Stm* or its isogenic ΔSPI1 mutant (MOI of 25 or 5 for neutrophils and macrophages, respectively), and cell extracts and supernatants were harvested at 5 hr postinfection. Cleaved caspase-1 and IL-1β in cell supernatants (SN) and expression of pro-caspase-1, pro-IL-1β, ASC, and GAPDH (loading control) in cell extracts (XT) were detected by western blot.

All ELISA data are mean + SD of technical triplicate cell stimulations. All data are representative of three independent experiments, except (B)–(D) data, which are representative of two independent experiments. See also Figure S3.

neutrophil IL-1β secretion at 5 hr postinfection (Figure 2E; Figures S3A and S3B). Western blot confirmed that IL-1β and caspase-1 are both efficiently processed, and their mature forms (IL-1β p17, caspase-1 p20) were released from WT neutrophils, but not *Nlrc4*$^{-/-}$ or caspase1/11-deficient *Ice*$^{-/-}$ neutrophils infected with WT *Stm* (Figure 2E). We next examined whether neutrophils secrete other caspase-1-dependent cytokines upon NLRC4 activation. IL-18 secretion by *Stm*-infected neutrophils and macrophages followed a similar pattern to IL-1β production by these cells (Figures S3B and S3C); however, LPS-primed neutrophils were poor producers of IL-18, as compared to IL-1β. Consistent with previous reports (Broz et al., 2010a; Man et al., 2014), we observed that *Nlrp3* deficiency led to a modest but reproducible suppression of IL-1β and IL-18 production in LPS-primed neutrophils and macrophages (Figures S3A–S3D). In examining this further, we observed that NLRP3 appeared to provide a supportive function to the neutrophil NLRC4 inflammasome at 1 hr postinfection, but the contribution of NLRP3 to NLRC4-dependent IL-1β production diminished as the time course progressed (Figure S3E). We confirmed that caspase-11 was dispensable for neutrophil IL-1β production under our experimental conditions (Figure S3F). Together, these data indicate that neutrophils can trigger NLRC4 function, wherein NLRC4-dependent caspase-1 drives the production of inflammasome target cytokines. IL-1β was the dominant cytokine secreted by this pathway in LPS-primed neutrophils, perhaps due to stronger intracellular expression of this cytokine precursor as compared to pro-IL-18.

The Neutrophil Inflammasome Is Activated during In Vivo Infection

We next examined whether, like bone marrow neutrophils challenged in vitro, neutrophils elicited and infected by *Stm* in vivo can produce inflammasome-dependent IL-1β. WT and *Ice*$^{-/-}$ mice were infected (i.p.) for 6 hr with 10^6 CFU *Stm*, after which elicited neutrophils were purified and cultured ex vivo in the presence of the membrane-impermeable antibiotic gentamicin to eliminate extracellular bacteria. Despite only low levels of active cell infection at these time points (Figure 3A), WT neutrophils efficiently secreted IL-1β ex vivo (Figure 3B), and IL-1β secretion was abrogated in *Ice*$^{-/-}$ mice, indicating that neutrophil IL-1β secretion was inflammasome dependent. Consistent with

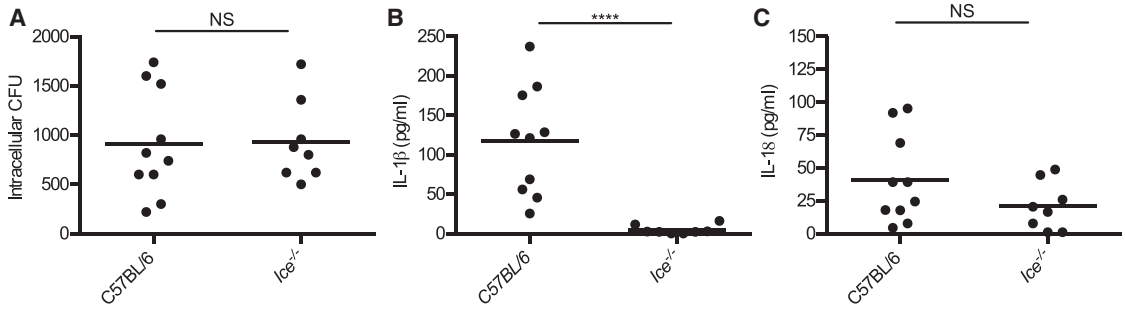


Figure 3. Neutrophils Infected with *S.* Typhimurium In Vivo Trigger Inflammasome-Dependent IL-1β Secretion

Mice were challenged with 10^6 cfu *Stm* and sacrificed at 6 hr postinfection. $Ly6G^+$ neutrophils in the peritoneal exudate were purified by positive selection.

(A) Neutrophil intracellular bacterial loads were analyzed at 0.5 hr postpurification.

(B and C) In vivo-elicited neutrophils were cultured ex vivo for 8 hr, and supernatants were collected for IL-1β (B) and IL-18 (C) quantification by ELISA.

Data are mean of neutrophils elicited from individual mice (eight to ten mice per genotype) combined from two independent experiments.

in vitro data (Figure S3C), purified neutrophils infected in vivo produced minimal caspase-dependent IL-18 (Figure 3C).

Neutrophils Are a Major Source of IL-1β during Acute *Salmonella* Infection

Given that neutrophils possess a functional NLRC4 inflammasome (Figure 2) and comprise a large component of the cellular infiltrate in many inflammatory settings, we hypothesized that the neutrophil compartment may be a significant contributor to IL-1β production during acute infection. We first measured the kinetics of immune cell recruitment during *Stm*-induced peritonitis. In uninfected mice, neutrophils ($CD11b^+Ly6C^+Ly6G^+$) and monocytes ($CD11b^+Ly6C^+Ly6G^-$) are barely detectable, and resident macrophages ($CD11b^+F4/80^+$) are the major $CD11b^+$ myeloid cells in the peritoneal cavity (Figures 4A and 4B). Neutrophils were recruited as early as 1 hr postinfection, and neutrophil numbers increased by 165-fold compared to uninfected mice at 6 hr (Figure 4B). Monocytes were recruited more slowly and weakly as compared neutrophils, showing significant recruitment at 4 hr (11-fold) and 6 hr (26-fold) as compared to uninfected mice (Figure 4B). In contrast, macrophage abundance in the peritoneal exudate declined throughout the infection time course, showing significantly decreased abundance at 4 and 6 hr (Figures 4A and 4B), consistent with previous reports (Gomez et al., 2012; Xu et al., 2009). We also profiled the kinetics of intracellular pro-IL-1β expression in myeloid cell subsets over the same infection time course. Macrophages, monocytes, and neutrophils all showed a rapid induction in intracellular pro-IL-1β expression in the first 2 hr of *Stm* challenge (Figure S4A).

To quantify the contribution of the neutrophil compartment to inflammasome-dependent IL-1β production in vivo, we examined the effect of neutrophil depletion. Twenty-four hours after α-Ly6G antibody injection, mice were challenged with *Stm* over 6 hr. α-Ly6G administration efficiently depletes neutrophils (Figure 4C) without affecting the abundance of other myeloid cells (Figures S4B and S4C), as shown previously (Daley et al., 2008; Franchi et al., 2012). Consistent with the strong expression of intracellular pro-IL-1β in macrophages ($CD11b^+F4/80^+$) 1 hr post *Stm* challenge (Figure S4A), IL-1β levels in the peritoneal lavage fluid dramatically increased at 1 hr postinfection in both undepleted and neutrophil-depleted mice (Figure 4D), suggesting that resident peritoneal macrophages provide the initial wave of IL-1β during *Stm* challenge. However, IL-1β levels continued to increase by 2-fold between 1 hr and 2 hr in undepleted mice (coinciding with neutrophil recruitment; Figure 4B), whereas IL-1β levels remained unchanged in depleted mice (Figure 4D). IL-1β levels remained higher (3-fold) in undepleted mice as compared neutrophil-depleted mice at 6 hr postinfection (Figure 4D). Importantly, neutrophil depletion did not affect monocyte/macrophage intracellular pro-IL-1β (Figures S4D and S4E), indicating that neutrophil depletion did not indirectly influence IL-1β expression or secretion from monocyte/macrophages. Collectively, these data suggest that resident macrophages provide the first wave of IL-1β production within the first hour of infection, whereas newly recruited neutrophils continue to produce IL-1β from 1 to 6 hr. Similar results were obtained 12 hr postinfection, where neutrophil depletion reduced IL-1β levels in the peritoneum by 60% (Figures S4F and S4G) and in the serum by 50% (Figure 4E). These results, coupled with our earlier data showing that in vivo-challenged neutrophils efficiently produce IL-1β ex vivo, suggest that neutrophils are a major cellular compartment for IL-1β production from 1 to 12 hr postinfection. As expected given the known antimicrobial functions of neutrophils during *Stm* infection, neutrophil-depleted mice exhibited significantly higher bacterial burdens in both liver and spleen (Figures 4F and 4G). Neutrophil-derived IL-1β likely contributes to host defense, as IL-1β neutralization increased bacterial burden in the liver and spleen (Figures 4H and 4I).

Consistent with our earlier observations that primed neutrophils are poor producers of IL-18 (Figure 3B; Figure S3), acute (1–6 hr) infection did not upregulate IL-18 levels in the peritoneum (Figure S5A), and neutrophil depletion did not significantly affect IL-18 production after 12 hr *Stm* challenge in vivo (Figure S5B), despite modest IL-18 induction at this time point (Figures S5C). Thus nonneutrophilic cells are the primary producers of IL-18 during acute *Stm* challenge.

Neutrophils Are Resistant to Pyroptosis In Vitro and In Vivo

In addition to the regulation of inflammasome-dependent cytokines, a hallmark of NLRC4 activation in macrophages and dendritic cells is the rapid induction of caspase-1-dependent but

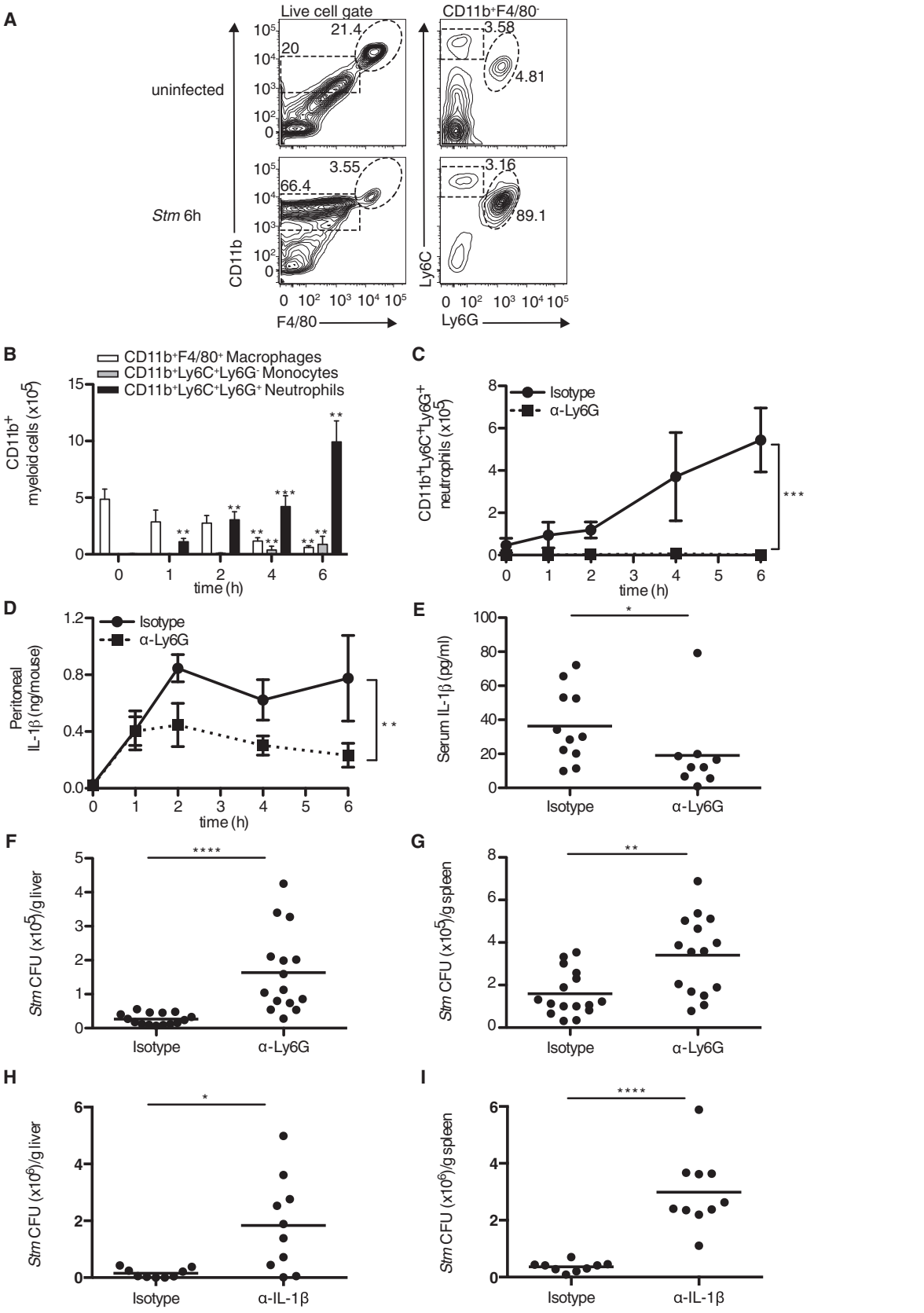
cytokine-independent pyroptotic cell death (Mariathasan et al., 2004). We thus examined whether *Stm* infection also triggered NLRC4-dependent neutrophil death in concert with cytokine regulation. As for previous experiments, we primed neutrophils and macrophages with LPS and then infected cells in vitro with increasing doses of *Stm*. Surprisingly, NLRC4-dependent caspase-1 activation (Figure 2) did not trigger concomitant neutrophil death, as assessed by light microscopy (not shown) or by quantifying the release of intracellular lactate dehydrogenase (LDH) into the supernatant (Figure 5A). By contrast, macrophages underwent rapid caspase-1-dependent cell death upon *Stm* infection (Figure 5B). Macrophage pyroptosis was evident as early as 1 hr postinfection, whereas neutrophils showed no evidence of caspase-1-dependent cell death as late as 5 hr (Figures 5A and 5B) or even 16 hr (Figure S6A) postinfection, despite clear neutrophil death when treated with an apoptotic trigger, staurosporine, at these time points (Figure S6B). In keeping with the lack of inflammasome-dependent cell death in neutrophils and the reported ASC independence of NLRC4/caspase-1-triggered pyroptosis in macrophages (Broz et al., 2010b), *Asc* deficiency did not affect LDH release in neutrophils or macrophages at 5 hr postinfection (Figures 5C and 5D). While NLRC4 and the *Stm* SPI1-T3SS were absolutely required for *Stm*-dependent cell death in macrophages, neither WT *Stm* nor its isogenic ΔSPI1 mutant triggered neutrophil cell death, and this was unaffected by knockout of inflammasome components (Figures S6C and S6D). To test whether the ability of neutrophils to resist caspase-1-dependent cell death was a general feature of inflammasome pathways in this cell type, we next examined pyroptotic cell death downstream of a non-NLR inflammasome. AIM2 is a cytosolic double-stranded DNA sensor of the HIN200 family (Cridland et al., 2012) and a potent driver of pyroptotic cell death in macrophages. Although AIM2 activation by transfected DNA enabled caspase-1 processing, it did not trigger caspase-1-dependent neutrophil death (Figure 5E). Together, these in vitro data indicate that NLRC4 activation in macrophages triggers rapid caspase-1-dependent cell death, whereas neutrophils are resistant to this arm of caspase-1 signaling, despite the concomitant activity of other caspase-1 functions (e.g., IL-1β processing; Figure 2).

Pyroptotic death is a host defense mechanism that restricts the ability of *Stm* to replicate intracellularly within macrophages (Miao et al., 2010a). As neutrophils were resistant to pyroptotic cell death, we hypothesized that unlike macrophages, caspase-1 would not serve to suppress the intracellular pathogen loads of neutrophils. We thus performed gentamicin protection assays on neutrophils and macrophages and monitored intracellular bacterial loads over time (Figures 5F and 5G). In line with previous studies (Miao et al., 2010a, 2010b), caspase-1 deficiency dramatically increased the intracellular bacterial burden of macrophages (Figure 5G), coinciding with their inability to undergo cell death, and both caspase-1-dependent and -independent mechanisms contributed to suppressing intracellular pathogen loads by 16 hr postinfection. By contrast, the intracellular bacterial burden of neutrophils was not affected by caspase-1/11 deficiency (Figure 5F), correlating with a lack of bacterial release by pyroptosis. The intracellular loads of live bacteria were lower in neutrophils versus macrophages, likely reflecting their superior short-term antimicrobial defense mechanisms, and we observed the presence of *Stm* in most neutrophils by microscopy (not shown). Surprisingly, both WT and *Ice*$^{-/-}$ neutrophils failed to suppress intracellular bacterial burden by 16 hr postinfection. In vivo, it is likely that a complex interaction of different cell types and cytokines promotes optimal killing of *Stm* by neutrophils, and this is not fully replicated in vitro. However, it is also possible that for microbes that can evade neutrophil-mediated killing, the lack of neutrophil pyroptosis generates a short-term niche for bacterial survival.

To investigate whether in vivo challenged neutrophils are also resistant to pyroptosis, we infected WT and *Ice*$^{-/-}$ mice with *Stm* intraperitoneally (i.p.) for 6 hr, purified the in vivo-infected neutrophils, and cultured them ex vivo for 8 hr. Despite inflammasome-dependent IL-1β release (Figure 3A), caspase-1/11 deficiency did not affect the release of intracellular LDH (Figure 6A). To further investigate this phenomenon in a fully in vivo setting, we challenged mice with *Stm* i.p. for 1.5 hr. As inflammasome-dependent cell death triggers a rapid loss in plasma membrane integrity, pyroptotic cells can be identified with the membrane-impermeable dye 7-AAD (Miao et al., 2010a). A total of 18% of resident peritoneal macrophages were no longer viable at 1.5 hr post *Stm* challenge, but macrophage death was 2-fold reduced in *Ice*$^{-/-}$ animals (Figure 6B). In contrast, 7-AAD uptake by neutrophils at the same time point was low, and slight differences in neutrophil death in WT versus *Ice*$^{-/-}$ animals were not statistically significant (Figure 6B). To ensure sufficient time for the activation of caspase-1-dependent programs in newly recruited neutrophils, the experiment was repeated using an extended infection time course of 6 hr. As for 1.5 hr (Figure 6B), 7-AAD uptake remained low among neutrophils at 6 hr postinfection and showed no significant difference between WT and *Ice*$^{-/-}$ neutrophils (Figure 6C). In all, our in vitro and in vivo data indicate that while neutrophils drive substantial inflammasome-dependent IL-1β release, they possess a modified inflammasome pathway that renders them resistant to pyroptotic cell death during challenge with *Stm.*

DISCUSSION

The NLRC4 inflammasome pathway is now well established to mediate host inflammatory and antimicrobial responses, leading to pathogen control and clearance. However, the key cell types responsible for initiating NLRC4 pathways during in vivo infection with *Stm* were unclear. During *Stm* intraperitoneal challenge, peritoneal macrophages quickly respond to *Stm* challenge with NLRC4 inflammasome formation and caspase-1 activation, culminating in rapid caspase-1-dependent IL-1β production and pyroptotic cell death. However, despite rapid macrophage lysis (apparent as early as 1.5 hr), peritoneal IL-1β levels continue to rise past 24 hr postinfection, suggesting the possibility that other cells may be able to signal via inflammasomes at a site of infection. Neutrophils predominate the early inflammatory infiltrate, presenting these cells as excellent candidates for driving NLRC4-dependent IL-1β production. Neutrophils were previously reported not to trigger NLRC4 inflammasome-dependent pathways, because they did not undergo pyroptotic cell death when infected with typical NLRC4 activators such as *Stm*



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(Ceballos-Olvera et al., 2011; Miao et al., 2010a); however, such studies did not assess the capacity for neutrophils to cleave caspase-1 or produce inflammasome-target cytokines.

Here, we demonstrate that *Stm* infection indeed triggers neutrophil NLRC4-dependent caspase-1 activation and IL-1β production in vitro and that neutrophils are a major compartment for inflammasome-dependent IL-1β production in vivo. By specifically depleting neutrophils in vivo prior to infection with *Stm*, we quantified the contribution of neutrophils to both the IL-1β response and pathogen burden during acute infection. Neutrophil depletion dramatically increased pathogen loads in liver and spleen, consistent with the important function of these cells in controlling murine *Stm* infection as is well established in intravenous, intraperitoneal, and oral challenge models (Cheminay et al., 2004; Conlan, 1996, 1997; Dejager et al., 2010; Franchi et al., 2012; Miao et al., 2010a; Seiler et al., 2000; Vassiloyanakopoulos et al., 1998). Our data suggest that after an initial burst of IL-1β release from resident macrophages, recruited neutrophils become the predominant source of secreted IL-1β from 1 to 12 hr postinfection. Neutrophil-derived IL-1β is thus likely to mediate a positive amplification loop driving further neutrophil recruitment and activation, presenting an autoregulatory paradigm for these cells during pathogen control. Of note, similar pathways may control neutrophil function downstream of other neutrophil-expressed inflammasomes, such as AIM2 and NLRP3. Three recent reports suggest that the NLRP3 inflammasome enables IL-1β processing in neutrophils, in vitro (Bakele et al., 2014; Mankan et al., 2012) and in vivo (Cho et al., 2012), whereas pyroptosis was not examined in any of these studies. We confirmed herein that, as for macrophages as was previously published (Broz et al., 2010a; Man et al., 2014), NLRP3 appears to collaborate with the NLRC4 inflammasome in neutrophils. The published literature for NLRP3 function in neutrophils, and our studies presented here characterizing neutrophil NLRC4, NLRP3, and AIM2 inflammasome function, collectively overturn the general assumption that neutrophils can only trigger IL-1β production via inflammasome-independent mechanisms such as Fas receptor ligation (Miwa et al., 1998) or cleavage via granular proteases (Karmakar et al., 2012). Indeed, in the *Stm* model studied here, neutrophil-derived IL-1β was entirely dependent on inflammasome-activated caspase-1. The inflammasome dependency of neutrophil IL-1β may depend on the microbial trigger, as neutrophils produced IL-1β independently of inflammasomes during *P. aeruginosa* infection (Karmakar et al., 2012).

The contribution of IL-1β to host defense in murine *Stm* challenge is somewhat controversial. In oral infection models, deficiency in *Il1β* or its receptor was associated with moderately increased susceptibility to *Stm* infection in BALB/c (Franchi et al., 2012) or C57BL/6 (Raupach et al., 2006) mice, although the latter is controversial (Franchi et al., 2012). For intraperitoneal infection, IL-1β/IL-1R appeared dispensable for pathogen control and mouse survival at 3 days postinfection, whereas the important protective functions of IL-18 in host defense were evident at this time point (Franchi et al., 2012; Raupach et al., 2006). Because IL-1β exerts a range of activities to promote neutrophil-mediated pathogen clearance in a number of other bacterial infection models (Chen and Schroder, 2013), and because our data indicate that IL-18 levels are unchanged in the acute (0–6 hr) phase of intraperitoneal challenge when neutrophils predominate, we investigated whether IL-1β contributes to pathogen control during acute infection. Indeed, significantly increased bacterial burdens were evident in the organs of IL-1β-neutralized mice at 12 hr postinfection. In light of previous studies, our data suggest a model in which macrophage- and neutrophil-derived IL-1β supports host defense in the acute phase of infection, whereas IL-18 production becomes important for microbial control in the latter stages of infection.

The neutrophil NLRC4 pathway is unique among cells described to date. Despite clear NLRC4-dependent caspase-1 activation, neutrophils did not undergo pyroptotic cell death, in vitro or in vivo. The resistance of neutrophils to pyroptotic cell death appears to allow sustained IL-1β release, as compared to macrophages where cytokine production plateaued within 3 hr, coinciding with cell lysis. Although our observation of caspase-1-dependent cytokine processing uncoupled from pyroptotic death identifies neutrophil signal specialization downstream of NLRs, specialization in other PRR signaling pathways (e.g., TLRs, CLRs) is already well documented (Thomas and Schroder, 2013). For example, TLR4 in macrophages elicits dual signaling pathways via MyD88 and TRIF, whereas TLR4 solely signals through MyD88 in human neutrophils (Tamassia et al., 2007).

Our in vivo data suggest a model in which resident macrophages and recruited neutrophils collaborate, together coordinating caspase-1-dependent inflammation and pathogen elimination. Resident peritoneal macrophages respond to infection by secreting a rapid burst of IL-1β within the first hour to initiate inflammation and neutrophil influx, whereas neutrophils are the dominant IL-1β-secreting cells from 1 to 12 hr post *Stm* challenge. A study from Miao and coworkers posits that macrophage pyroptosis is an innate defense mechanism that prevents *Stm* replication within macrophages, and allows bacterial release

Figure 4. Neutrophils Are Major Producers of IL-1β during Acute *Salmonella* Infection

(A and B) Flow cytometry plots (A) and absolute abundance (B) of macrophages ($CD11b^+F4/80^+$), neutrophils ($CD11b^+Ly6C^+Ly6G^+$), and monocytes ($CD11b^+Ly6C^+Ly6G^-$) following i.p. infection with *Stm* (10^6 cfu). The statistical significance for each cell type at each time point was calculated relative to uninfected mice.

(C–G) Mice were administered with isotype control (mock depleted) or α-Ly6G antibody (neutrophil depleted) for 24 hr before infection with *Stm* (10^6 cfu). Neutrophil abundance in the peritoneum (C) and IL-1β levels in the peritoneal lavage fluid (D) up to 6 hr postinfection. At 12 hr postinfection, serum IL-1β was measured by ELISA (E), and pathogen loads in the liver (F) and spleen (G) were quantified by serial dilution.

(H and I) Mice were administered with isotype control or α-IL-1β neutralizing antibody for 16 hr prior to infection with *Stm*. At 24 hr postinfection, pathogen loads in the (H) liver and (I) spleen were quantified by serial dilution.

Flow cytometry plot in (A) is representative of at least four independent experiments. Data are mean (B–D) and SEM of three to eight individual mice per time point combined from two or three independent experiments (B–D), 14–16 mice pooled from three independent experiments (E–G), or nine or ten mice pooled from two independent experiments (H and I). See also Figures S4 and S5.

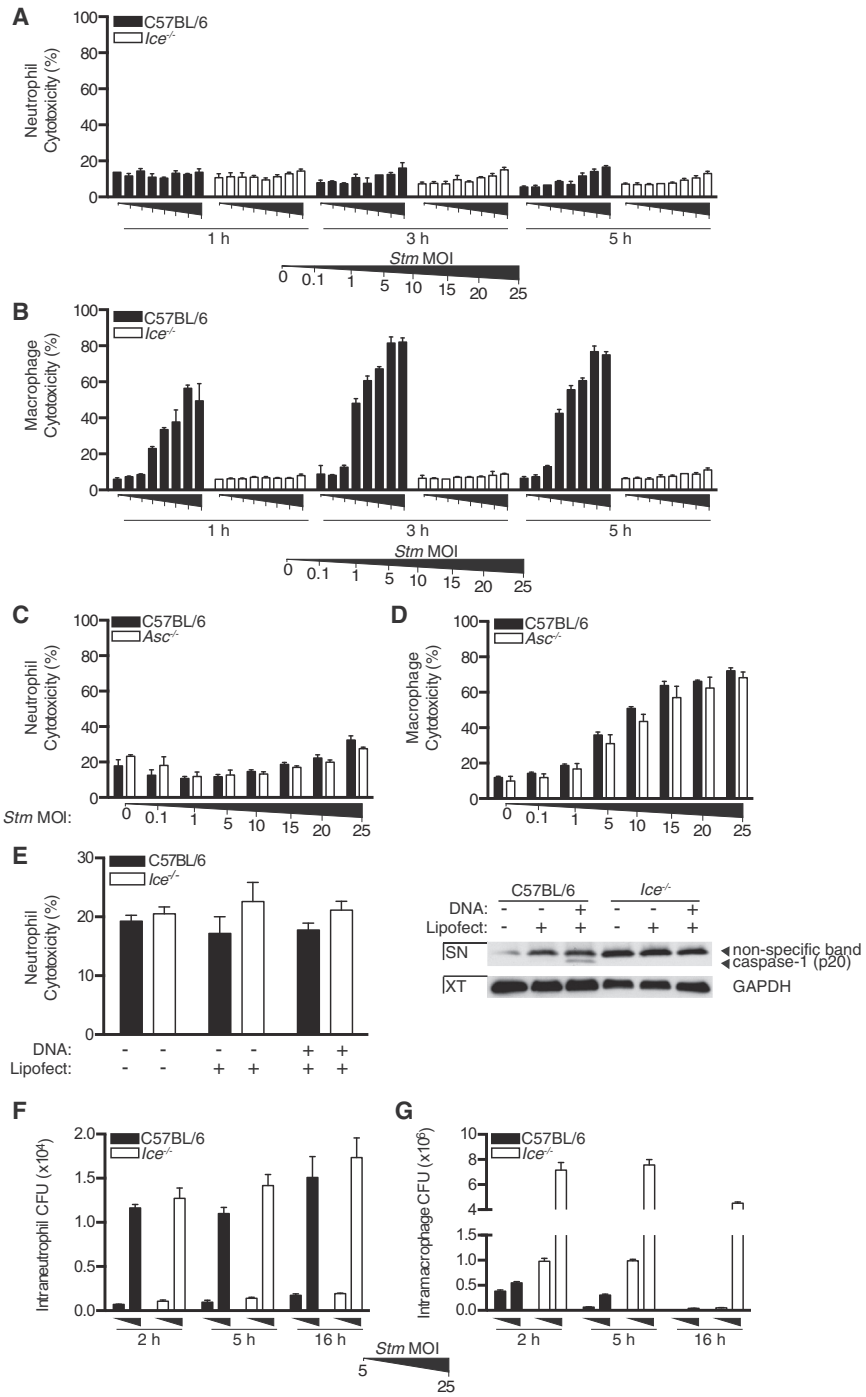

Figure 5. Neutrophils Do Not Undergo *Salmonella*-Dependent Pyroptosis In Vitro

Cytoplasmic LDH release into the supernatant, as compared to total intracellular LDH of uninfected cells was quantified as a measure of cell death.

(A–G) Bone marrow neutrophils (A, C, and F) and bone marrow-derived macrophages (B, D, and G) were all primed for 4 hr with LPS before infection with *Stm*. Cells were infected with increasing *Stm* doses, and LDH release was measured at 1, 3, and 5 hr (A and B) or 5 hr postinfection (C and D).

(E) Cells were LPS-primed for 3 hr before lipofectamine transfection of calf thymus DNA. LDH release was measured at 6 hr posttransfection. Caspase-1 cleavage was measured in cell supernatants (SN) by immunoblotting, relative to expression of the GAPDH loading control in cell extracts (XT).

(F and G) Cells were LPS-primed for 4 hr and infected with *Stm* (MOI 5 or 25). Intracellular bacterial CFU of bone marrow neutrophils (F) and bone marrow-derived macrophages (G) were enumerated.

Data are mean + SD of technical triplicate cell stimulations and are representative of three (A and B) or two (C–G) independent experiments.

for more effective killing by neutrophils (Miao et al., 2010a). Since neutrophils are well established as the primary cells mediating *Stm* elimination, the lack of neutrophil pyroptosis we observe makes intuitive sense, as microbial destruction rather than cell lysis is the appropriate response for a neutrophil encountering *Stm.* If neutrophils could not resist pyroptosis, their ability to eliminate *Stm* through classic antimicrobial mechanisms (reactive oxygen species, degranulation, phagocytosis, etc.) would be severely compromised, leading to pathogen dissemination. Although probably necessary to ensure pathogen clearance in the short term, the lack of a functional pyroptotic pathway in neutrophils, coupled with the extended lifespan of neutrophils in inflammatory microenvironments (Croker et al., 2012; Thomas and Schroder, 2013), may ultimately render these cells susceptible to intracellular pathogens. Indeed, several reports suggest that *Salmonella* (Dunlap et al., 1992; Geddes et al., 2007) as well as other pathogens such as *Neisseria gonorrhoeae* (Casey et al., 1986), *Staphylococcus aureus* (Rogers and Tompsett, 1952), *Chlamydia pneumoniae* (van Zandbergen et al., 2004), *Burkholderia pseudomallei* (Ceballos-Olvera et al., 2011), and *Anaplasma phagocytophilum* (Chen et al., 1994) reside and replicate efficiently within neutrophils in vivo and may use neutrophils to acquire nutrients, evade the immune system, and disseminate to other tissues. Indeed, our observations from neutrophil infection in vitro suggest that their resistance to pyroptosis may contribute to the inability of neutrophils to restrict intracellular *Stm* survival, similar to caspase-1-deficient macrophages.

The surprising lack of neutrophil pyroptosis has fundamental implications for our understanding of inflammasome pathways. First, it suggests that the caspase-1-dependent pathways mediating cytokine processing versus death are mechanistically

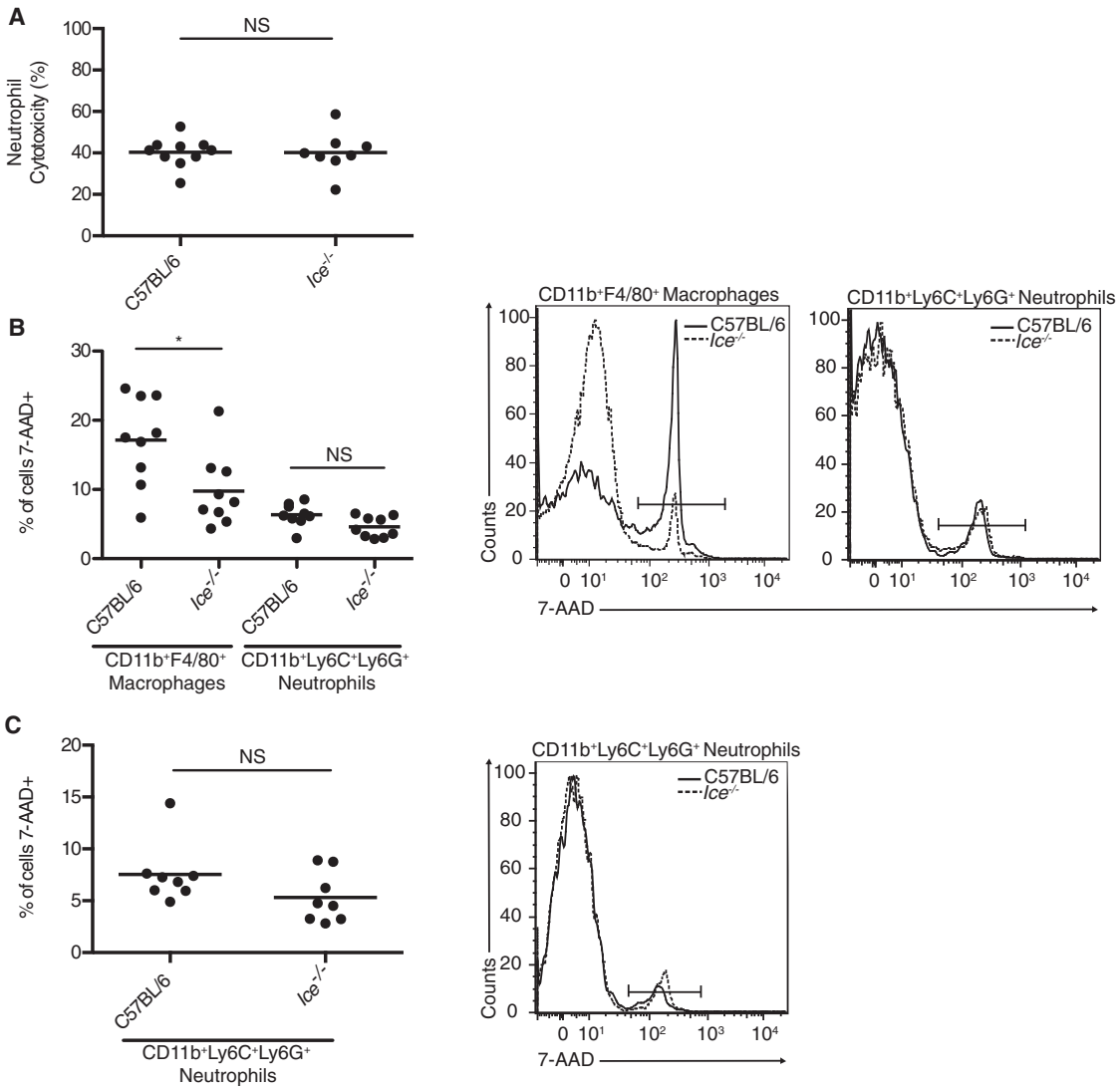

Figure 6. Neutrophils Infected with *Salmonella* In Vivo Resist Pyroptotic Cell Death

(A) Mice were challenged with *Stm* (10^6 cfu), and Ly6G$^+$ neutrophils in the peritoneal exudate at 6 hr postinfection were purified by positive selection. In vivo-elicited neutrophils were cultured ex vivo for 8 hr in the presence of 50 μg/ml gentamicin and assayed for LDH release. Data are mean of neutrophils elicited from eight to ten individual mice combined from two independent experiments.

(B and C) Percentage of 7-AAD$^+$ cells among macrophages or neutrophils in the peritoneal exudate following *Stm* challenge for 1.5 hr (B) or 6 hr (C) and representative flow cytometry plots. Data are mean of eight to nine individual mice combined from two independent experiments.

separable, in support of an earlier proposal that two distinct caspase-1 activation states drive cytokine processing versus lytic death (Broz et al., 2010b). Until caspase-1-dependent cell death programs are elucidated, the mechanism by which neutrophils resist this process will remain elusive, but a likely scenario is that a caspase-1 proteolytic substrate required for the initiation of lytic cell death is not expressed in neutrophils. The finding that neutrophils have uncoupled IL-1β release from pyroptosis also unequivocally confirms the existence of caspase-1-dependent unconventional protein secretion pathways, refuting the alternative possibility that IL-1β is passively released during pyroptosis (Bortoluci and Medzhitov, 2010). Furthermore, the extended kinetics of IL-1β production in neutrophils as compared to macrophages suggests that pyroptotic cell death actually functions to curtail macrophage IL-1β processing and release.

In summary, our data indicate that neutrophils possess a unique inflammasome pathway that resists caspase-1-directed lytic cell death, thereby permitting extended cytokine production. Our finding that neutrophils are major contributors to inflammasome-dependent IL-1β during acute *Stm* infection may actually underestimate the importance of neutrophil inflammasomes in human infection and disease, as neutrophils are grossly underrepresented in murine as compared to human blood (Mestas and Hughes, 2004). NLRP3/12 dysfunction is associated with genetic and acquired human inflammatory diseases, including

hereditary fever syndromes and gout (Schroder and Tschopp, 2010), and these diseases are currently attributed to inflammasome dysfunction in monocytes/macrophages, in which cytokine production is intrinsically linked to cell death. This raises the intriguing question of why IL-1β production and inflammation are not self-limiting in these diseases, as one might expect IL-1β release to be transient if IL-1β-producing cells rapidly lyse. Our demonstration that human neutrophils express a range of inflammasome scaffolds and that neutrophil IL-1β production proceeds without restriction by inflammasome-dependent cell death pathways suggests that neutrophils may be critical cellular drivers of pathogenic IL-1β in human inflammatory disease.

EXPERIMENTAL PROCEDURES

Bacterial Strains

Stm SL1344 strains were grown at 37°C in Luria-Bertani medium at 200 rpm. The SL1344 ΔSPI1 (Δ*invA*) mutant was previously described (Kupz et al., 2012). Overnight bacteria were diluted 1:40 and grown for 3 hr to induce SPI1-T3SS expression.

Mice

Mice were backcrossed at least ten times to C57BL/6, and all in vivo experiments were conducted with age- and sex-matched mouse cohorts. C57BL/6, *Nlrc4*$^{-/-}$ (Mariathasan et al., 2004), *Nlrp3*$^{-/-}$ (Martinon et al., 2006), *Asc*$^{-/-}$ (Mariathasan et al., 2004), *Ice*$^{-/-}$ (Kuida et al., 1995), and *Casp11*$^{-/-}$ (Wang et al., 1998) mice were housed in specific-pathogen-free facilities at the University of Queensland. The University of Queensland's animal ethics committee approved all experimental protocols.

Preparation of Human and Mouse Cells

Fresh human peripheral blood was subject to discontinuous density sedimentation (Histopaque 1119 and 1077, Sigma) to separate neutrophils and eosinophils (densities >1.077 g/ml; PMN fraction) from cells with lower densities (PBMCs). CD16$^+$ neutrophils were further purified from the PMN fraction, and CD14$^+$ monocytes, CD4$^+$ T cells, CD19$^+$ B cells, and CD56$^+$ NK cells were purified from the PBMC fraction by magnetic-assisted cell sorting (MACS), according to standard protocols (Miltenyi Biotec). Monocyte-derived dendritic cells and monocyte-derived macrophages were differentiated for 7 days with granulocyte-macrophage colony-stimulating factor (100 ng/ml) plus IL-4 (25 ng/ml) or macrophage colony-stimulating factor (M-CSF; 100 ng/ml), respectively (all Immunotools). All MACS-purified cell preparations were assayed for purity by flow cytometry. Preparations of CD16$^+$, CD14$^+$, and CD3$^+$ cells achieved 97%–99% purity, whereas less abundant cell types were enriched (55% CD19$^+$ B cells; 70% CD56$^+$ natural killer cells). All mouse tissues were prepared as single-cell suspensions. Bone marrow cells and splenocytes were subject to erythrocyte lysis prior to fractionation. Splenocytes were stained with B220 and CD3, and B and T lymphocytes were enriched using standard MACS-positive selection techniques (Miltenyi Biotec). Bone marrow and elicited neutrophils were MACS purified using α-Ly6G-FITC (NIMP-R14 or 1A8) antibody and α-FITC beads. Bone marrow and elicited neutrophil populations achieved >98% purity, as assessed by flow cytometry. Splenic B and T cells were typically enriched to 88% and 64% purity, respectively. Bone marrow-derived macrophages and dendritic cells were differentiated as previously described (Gross, 2012; Schroder et al., 2012a).

mRNA Expression Profiling

Quantitative PCR was performed normalized to a human and mouse reference gene (HPRT) as previously described (Schroder et al., 2012a). All primer sequences are available on request. Primer pairs flanked exon-exon junctions to avoid amplification of contaminating genomic DNA, and primer pair efficiencies were quantified and used in calculations to generate cDNA profiles.

Neutrophil and Macrophage In Vitro Infection Assays

BMNs were used for experiments on the day of purification and were plated in RPMI-1640 supplemented with 10% fetal calf serum (FCS), 10 mM HEPES (all Life Technologies), and 0.4 μg/ml aprotinin (Sigma) at a density of 3.3 × 10^6 cells/ml, except experiments with ΔSPI1 bacteria that were plated at 2 × 10^7 neutrophils/ml. Differentiated BMDMs were cultured at a density of 0.8 × 10^6 cells/ml in RPMI-1640 supplemented with 10% FCS, 1x Glutamax (Life Technologies), and 10^4 U/ml recombinant human M-CSF (a gift from Chiron). BMNs or BMDMs were primed with 100 ng/ml ultrapure *E. coli* K12 LPS (Invivogen) for 4 hr to induce the expression of pro-IL-1β. BMNs and BMDMs were infected at the indicated MOI and centrifuged immediately at 700 g for 10 min at room temperature. Cells were incubated at 37°C for 25 min to allow phagocytosis of extracellular bacteria. Complete media was then replaced with Opti-MEM (Life Technologies) supplemented with 50 μg/ml gentamicin (Life Technologies) for the remainder of the assay to kill extracellular bacteria. Cells were then further incubated for the indicated times before harvesting cell-free supernatants and cell extracts for cytokine and LDH release assays. For the calculation of live intracellular bacteria, 25 min after spin infection, cells were washed thrice with complete media containing 200 μg/ml gentamicin and cultured for a further 30 min to kill extracellular bacteria. Cells were then cultured in complete media containing 20 μg/ml gentamicin for up to 16 hr before determination of intracellular bacterial loads.

In Vivo Infection and Neutrophil Depletion

Neutrophils were depleted or mock-depleted by i.p. administration of 0.1 mg α-Ly6G antibody (endotoxin-free 1A8 clone, BioXcell) or isotype control antibody (endotoxin-free 2A3 clone, BioXcell). Then 24 hr after antibody injection, mice were challenged with i.p. 1 × 10^6 colony-forming unit (cfu) log-phase *Stm* SL1344. For IL-1β neutralization experiments, mice were administered 0.1 mg IL-1β neutralizing antibody (endotoxin-free B122 clone, BioXcell) or isotype antibody (endotoxin-free, polyclonal hamster immunoglobulin G, BioXcell) i.p. 16 hr prior to *Stm* challenge. Mice were sacrificed at 1–24 hr postinfection as indicated, and the peritoneal cavity was flushed with 10 ml of DPBS. Cytokine levels and cellularity in the peritoneal lavage fluid were analyzed by ELISA and flow cytometry, respectively. Liver and spleen were homogenized, and bacterial loads were determined using serial dilution. To culture in vivo-challenged neutrophils ex vivo, neutrophils were purified from the peritoneal exudate using α-Ly6G MACS and cultured at 1.6 × 10^6 cells/ml for a further 8 hr in Opti-MEM supplemented with 50 μg/ml gentamicin. Bacterial loads were quantified by serial dilution following 0.5 hr incubation with 50 μg/ml gentamicin.

Flow Cytometry

Myeloid cells were identified using α-CD11b (M1/70), macrophages were labeled with α-F4/80 (BM8), neutrophils and monocytes were discriminated by α-Ly6G (1A8) and α-Ly6C (HK1.4) staining, and T and B cells were labeled with α-CD3 (17A2) and α-B220 (RA3-6B2), respectively (all BioLegend). Intracellular pro-IL-1β was detected using the NJTEN3 antibody (eBioscience). Dead cells were labeled with 7-AAD (Becton Dickinson). Cell profiles were acquired using a BD Canto II and analyzed using FlowJo (Tree Star).

Inflammasome and Pyroptosis Assays

IL-1β (R&D Systems) and IL-18 (MBL, eBioscience) levels in cell-free supernatants and serum were analyzed by ELISA. For AIM2 inflammasome activation studies, neutrophils (10^6) were transfected with 0.125 μg calf thymus DNA using Lipofectamine 2000. Western blots were performed on cell extracts and methanol/chloroform-precipitated cell-free supernatants as previously described (Gross, 2012). Antibodies included IL-1β (polyclonal goat, R&D Systems), caspase-1 (Casper-1, Adipogen), ASC ([N-15]-R, Santa Cruz Biotechnology), and GADPH (polyclonal mouse, BioScientific). Cytotoxicity was analyzed by LDH release (TOX7, Sigma).

Statistical Analysis

Statistical analyses were performed using the nonparametric Mann-Whitney t test or a two-way ANOVA using Prism GraphPad software. Data were considered significant when $p \leq 0.05$ (*), 0.005 (**), 0.001(***), or 0.0001(****).

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.06.028.

AUTHOR CONTRIBUTIONS

K.W.C. performed all experiments except expression profiling studies, which were performed by C.J.G. and K.S. F.V. provided technical assistance. K.J.S., J.T., and M.J.S. provided reagents and intellectual input. K.S. supervised the study. K.W.C. and K.S. wrote the manuscript, which all authors reviewed before submission.

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Cell Reports, Volume *8*

Supplemental Information

The Neutrophil NLRC4 Inflammasome Selectively Promotes IL-1β Maturation without Pyroptosis during Acute *Salmonella* Challenge

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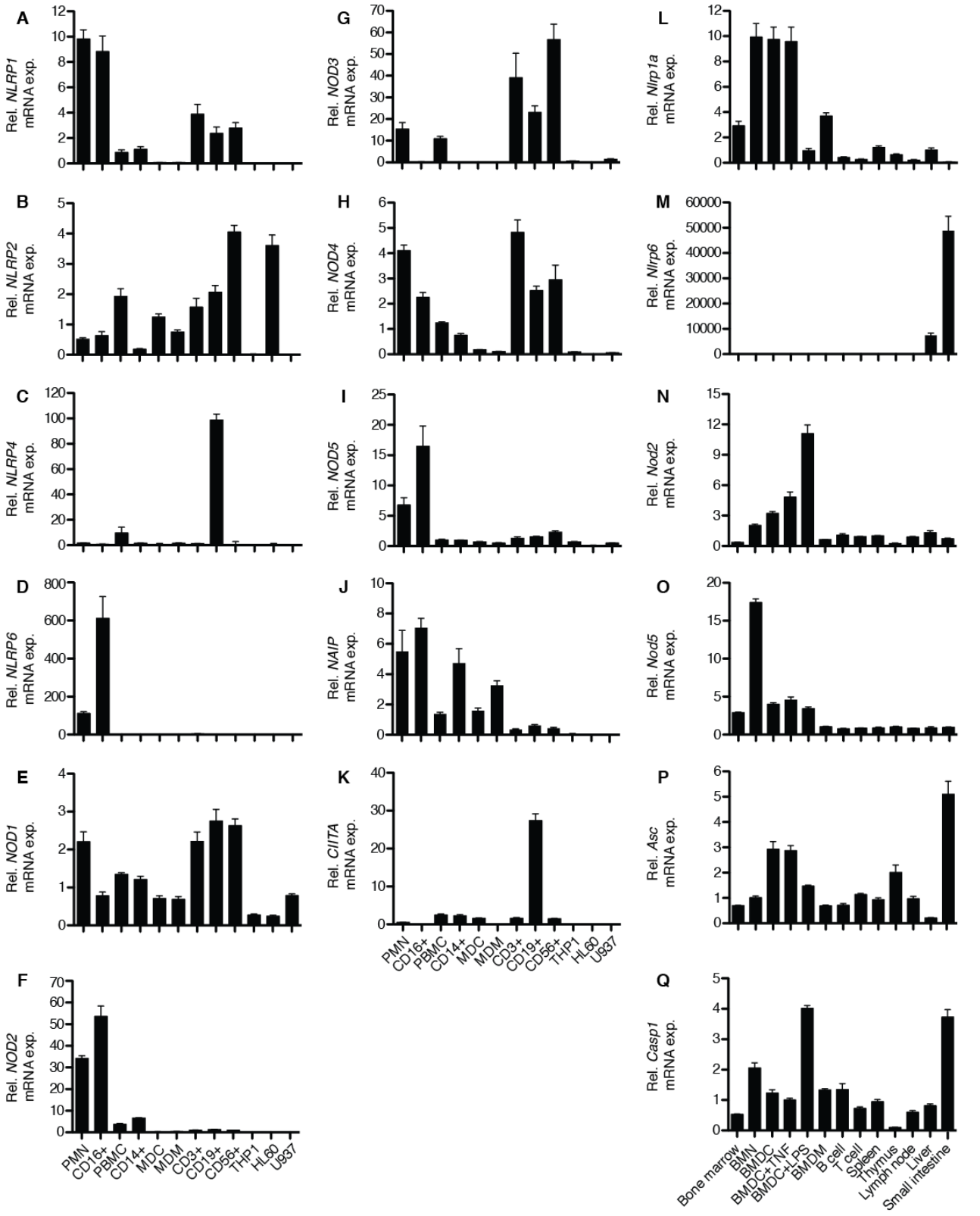
A
Rel. NLRP1 mRNA exp.
B
Rel. NLRP2 mRNA exp.
C
Rel. NLRP4 mRNA exp.
D
Rel. NLRP6 mRNA exp.
E
Rel. NOD1 mRNA exp.
F
Rel. NOD2 mRNA exp.
G
Rel. NOD3 mRNA exp.
H
Rel. NOD4 mRNA exp.
I
Rel. NOD5 mRNA exp.
J
Rel. NAIP mRNA exp.
K
Rel. CIITA mRNA exp.
L
Rel. Nlrp1a mRNA exp.
M
Rel. Nlrp6 mRNA exp.
N
Rel. Nod2 mRNA exp.
O
Rel. Nod5 mRNA exp.
P
Rel. Asc mRNA exp.
Q
Rel. Casp1 mRNA exp.
PMN
CD16+
PBMC
CD14+
MDC
MDM
CD3+
CD19+
CD56+
THP1
HL60
U937
Bone marrow
BMN
BMDC
BMDC+TNF
BMDC+LPS
BMDM
B cell
T cell
Spleen
Thymus
Lymph node
Liver
Small intestine
Human cells
Mouse cells and tissues

Figure S1. Expression profile detail for NLRs expressed in purified human and mouse leukocyte populations, cell lines and tissues, related to Figure 1. mRNA expression of **(A)** *NLRP1*, **(B)** *NLRP2,* **(C)** *NLRP4*, **(D)** *NLRP6*, **(E)** *NOD1*, **(F)** *NOD2*, **(G)** *NOD3*, **(H)** *NOD4*, **(I)** *NOD5*, **(J)** *NAIP*, and **(K)** *CIITA* was quantitated by qPCR in primary human blood populations: polymorphonuclear fraction (PMN; containing neutrophils and eosinophils), neutrophils ($CD16^+$ cells from PMN fraction, which excludes monocytes), bulk peripheral blood mononuclear cell fraction (PBMC), monocytes ($CD14^+$ cells from PBMC fraction), $CD14^+$ monocyte-derived dendritic cells (MDC), $CD14^+$ monocyte-derived macrophages (MDM), $CD3^+$ T cells, $CD19^+$ B cells and $CD56^+$ NK cells. The myeloid cell lines, THP-1, HL60 and U937 were also included on the panel. Data are mean + SD of technical triplicates and is representative of three experiments with independent blood donors. NLRs that were highly expressed in human neutrophils were examined for expression in mouse immune cell subsets and tissues by qPCR: **(L)** *Nlrp1a,* **(M)** *Nlrp6*, **(N)** *Nod2,* and **(O)** *Nod5*. mRNAs for the inflammasome components **(P)** *Asc* and **(Q)** *Casp1* were also measured in parallel. Cell populations are: BMN, bone marrow neutrophils; BMDC, bone marrow dendritic cells left untreated or treated overnight with TNF (25 ng/ml) or LPS (50 ng/ml); BMDM, bone marrow derived macrophages; B cells, $B220^+$ splenocytes; T cells, $CD3^+$ splenocytes. Data are mean + SD of technical triplicates and are representative of three independent experiments.

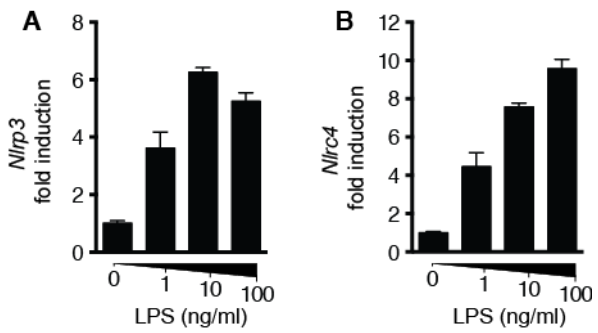

Figure S2. Neutrophil *Nlrp3* and *Nlrc4* expression is induced by LPS, related to Figure 1. BMN were treated with an increasing dose of LPS for 4 h and mRNA expression was quantified by qPCR. Data are mean + SD of technical triplicates.

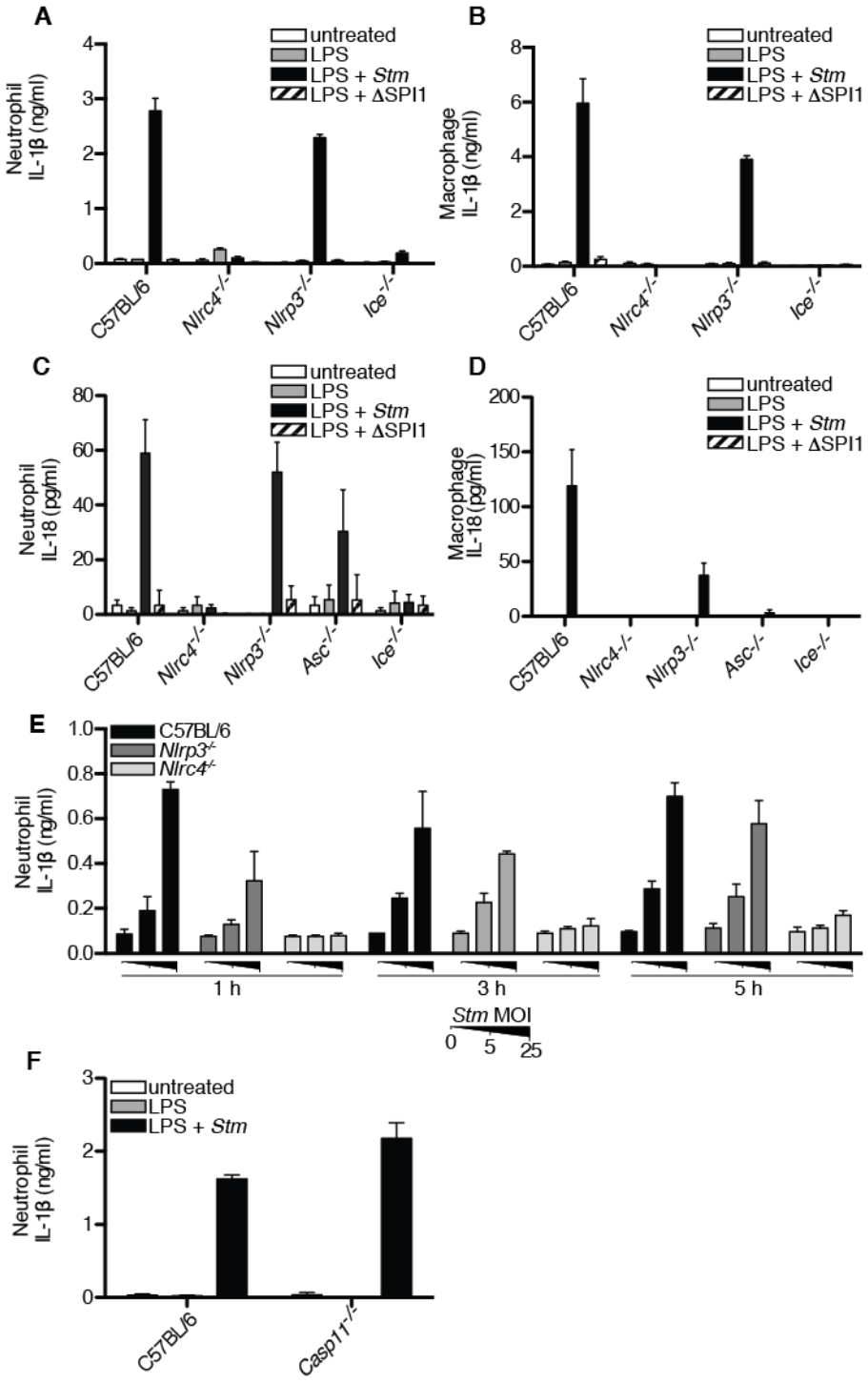

Figure S3. NLRP3 collaborates with the NLRC4 inflammasome during *Salmonella* infection, related to Figure 2. BMNs or BMDMs were treated with 100 ng/ml LPS before infection with *S*. Typhimurium (*Stm*) or its isogenic ΔSPI1 mutant. Neutrophils (**A, C, F**) and macrophages (**B, D**) were infected (MOI of 25 or 5 for neutrophils and macrophages, respectively) for 5 h and IL-1β (**A-B, F**) and IL-18 (**C-D**) release were measured by ELISA. (**E**) Neutrophils were infected with

increasing doses of *Stm* and IL-1β secretion was measured by ELISA at 1, 3 and 5 h post-infection. Data are (**A-B**) mean + SD of technical triplicate cell stimulations and are representative of (**A**) 3 or (**B**) 2 individual experiments, **(C)** mean + SEM of 3 independent experiments, (**D**) mean + SD of technical triplicate cell stimulations and are representative of 3 independent experiments, **(E)** mean + SD of technical triplicate cell stimulations and are representative of 2 independent experiments, (**F**) mean + SD of technical triplicate cell stimulations.

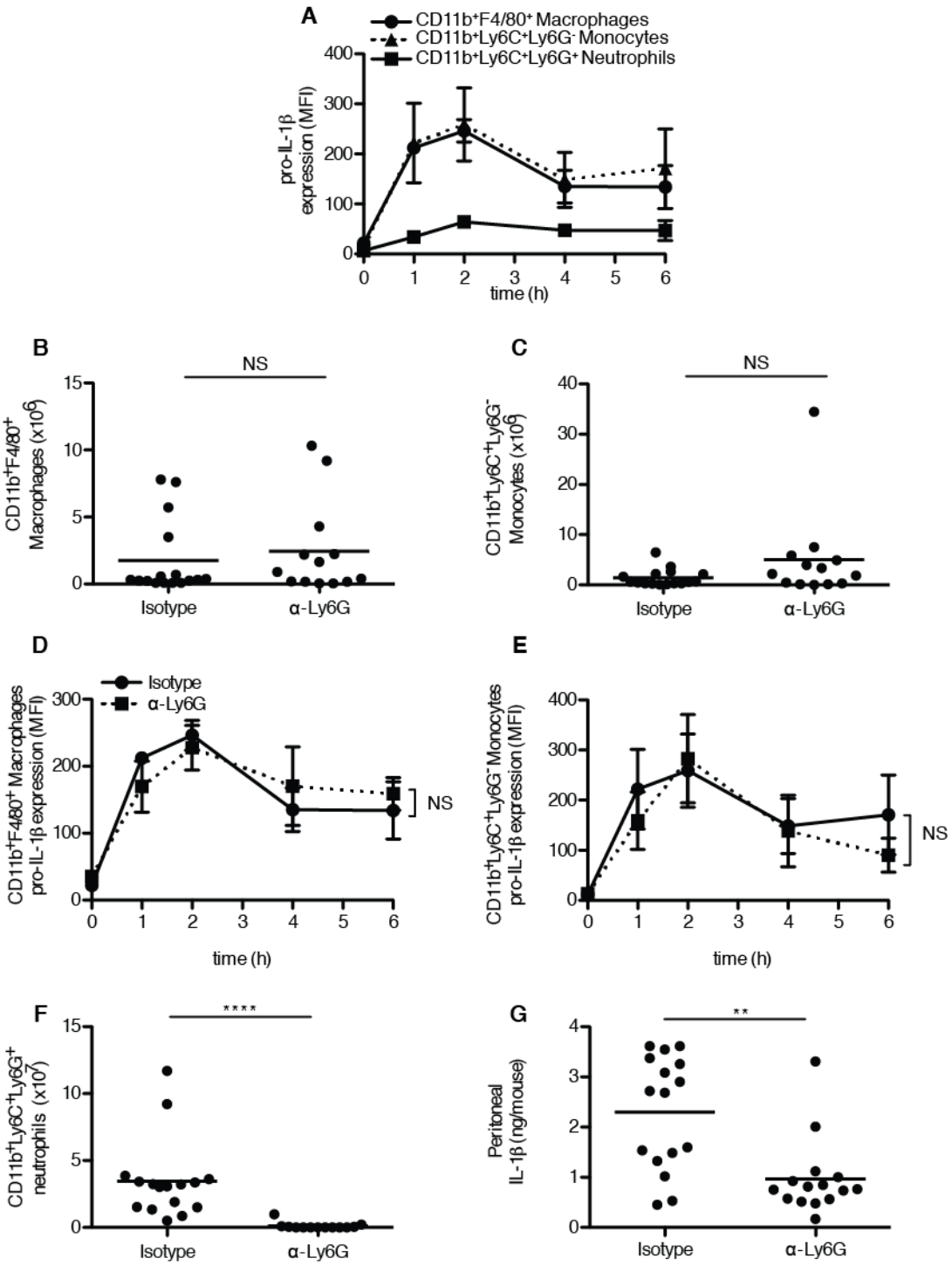
A
CD11b+F4/80+ Macrophages
CD11b+Ly6C+Ly6G- Monocytes
CD11b+Ly6C+Ly6G+ Neutrophils
pro-IL-1β expression (MFI)
time (h)
B
NS
CD11b+F4/80+ Macrophages (x10^6)
Isotype
α-Ly6G
C
NS
CD11b+Ly6C+Ly6G- Monocytes (x10^6)
Isotype
α-Ly6G
D
Isotype
α-Ly6G
CD11b+F4/80+ Macrophages pro-IL-1β expression (MFI)
NS
time (h)
E
CD11b+Ly6C+Ly6G- Monocytes pro-IL-1β expression (MFI)
NS
time (h)
F
****
CD11b+Ly6C+Ly6G+ neutrophils (x10^7)
Isotype
α-Ly6G
G
**
Peritoneal IL-1β (ng/mouse)
Isotype
α-Ly6G

Figure S4. Neutrophil depletion does not affect the abundance or intracellular pro-IL-1β expression of macrophages or monocytes, related to Figure 4. (A) Mice were challenged with i.p. *Stm* (10^6 CFU) and the peritoneal exudate was collected up to 6 h post-infection. Expression of intracellular pro-IL-1β in myeloid cells was measured by flow cytometry (mean fluorescence intensity). Data are mean ± SEM of 3-4 individual mice per time point combined from 2 independent experiments. **(B-G)** Mice were administered with isotype control (mock-depleted) or α-Ly6G antibody (neutrophil-depleted) for 24 h before infection with *Stm*. The abundance of (**B**) macrophages and (**C**) monocytes in the peritoneal exudate at 12 h post-infection was assessed by cell counting and flow cytometry. Data are mean of 14-16 mice pooled from 3 independent experiments. The intracellular expression of pro-IL-1β in peritoneal exudate (**D**) macrophages and (**E**) monocytes over a time course of infection was assessed by flow cytometry. Data are mean ± SEM of 3-5 individual mice per time point combined from 2 independent experiments. **(F)** Neutrophil abundance and **(G)** IL-1β levels in the peritoneal lavage fluid were also measured at 12 h post-infection. Data are mean of 14-16 mice pooled from 3 independent experiments.

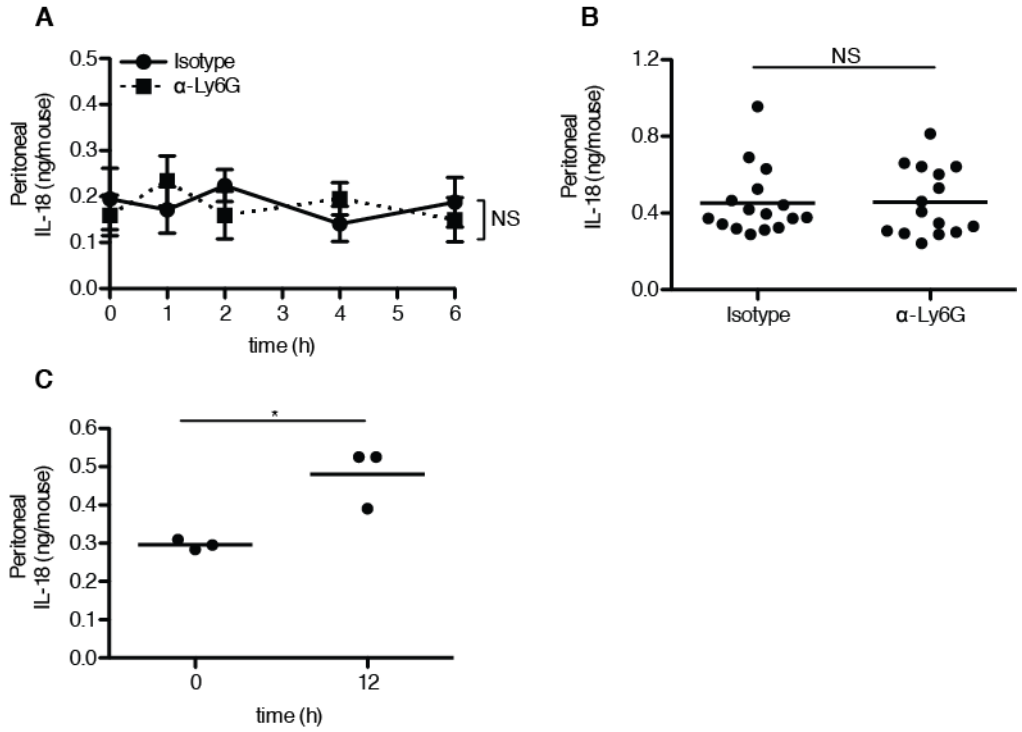

Figure S5. IL-18 is produced from 6-12 h post infection by non-neutrophilic cells, related to Figure 4. Peritoneal IL-18 levels in mice **(A-B)** administered isotype versus α-Ly6G antibody for 24 h or **(C)** not administered antibody before *Stm* challenge (10^6 CFU) for **(A)** 0-6 or **(B, C)** 12 h before quantitation of peritoneal IL-18 by ELISA. Data are mean ± SEM of (**A**) 3-5 individual mice per time point, combined from 2 independent experiments, mean of **(B)** 14-16 mice pooled from 3 independent experiments, or **(C)** 3 individual mice.

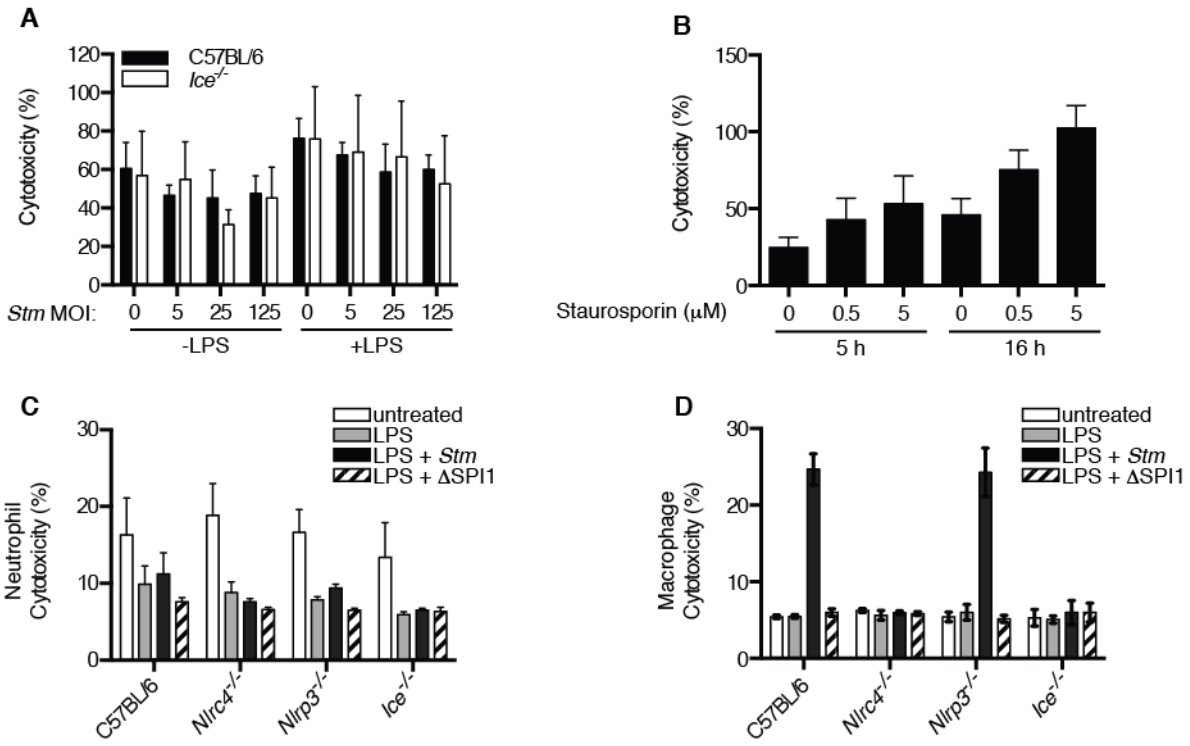

Figure S6. Neutrophils are resistant to pyroptotic cell death, related to Figure 5. (**A**) BMNs were plated in complete media and primed for 4 h with LPS or left untreated before infection with increasing doses of *Stm* for 16 h. (**B**) BMNs were treated with increasing concentrations of staurosporin to trigger apoptotic cell death, for 5 h and 16 h. (**C-D**) Cells were primed for 4 h with LPS and infected with *Stm* or its isogenic mutant (MOI of 25 or 5 for neutrophils and macrophages, respectively) and LDH release was measured at 1 h post-infection. Cytoplasmic LDH release into the supernatant, as compared to total intracellular LDH, was quantified as a measure of cell death. (**A-B**) Data are mean + SD of quadruplicate cell stimulations or (**C-D**) mean + SD of technical triplicate cell stimulations and are representative of 3 independent experiments.