Type 1 interferon-dependent repression of NLRC4 and iPLA2 licenses down-regulation of *Salmonella* flagellin inside macrophages

Ajay Suresh Akhade[a], Shaikh M. Atif[b,1], Bhavana S. Lakshmi[a], Neha Dikshit[b], Kelly T. Hughes[c], Ayub Qadri[b,2], and Naeha Subramanian[a,d,e,2]

[a]Institute for Systems Biology, Seattle, WA 98109; [b]Hybridoma Laboratory, National Institute of Immunology, 110067 New Delhi, India; [c]Department of Biology, University of Utah, Salt Lake City, UT 84112; [d]Department of Immunology, University of Washington, Seattle, WA 98109; and [e]Department of Global Health, University of Washington, Seattle, WA 98109

Edited by Vishva M. Dixit, Genentech, San Francisco, CA, and approved October 14, 2020 (received for review February 13, 2020)

Inflammasomes have been implicated in the detection and clearance of a variety of bacterial pathogens, but little is known about whether this innate sensing mechanism has any regulatory effect on the expression of stimulatory ligands by the pathogen. During infection with *Salmonella* and many other pathogens, flagellin is a major activator of NLRC4 inflammasome-mediated macrophage pyroptosis and pathogen eradication. *Salmonella* switches to a flagellin-low phenotype as infection progresses to avoid this mechanism of clearance by the host. However, the host cues that *Salmonella* perceives to undergo this switch remain unclear. Here, we report an unexpected role of the NLRC4 inflammasome in promoting expression of its microbial ligand, flagellin, and identify a role for type 1 IFN signaling in switching of *Salmonella* to a flagellin-low phenotype. Early in infection, activation of NLRC4 by flagellin initiates pyroptosis and concomitant release of lysophospholipids which in turn enhance expression of flagellin by *Salmonella* thereby amplifying its ability to elicit cell death. TRIF-dependent production of type 1 IFN, however, later represses NLRC4 and the lysophospholipid biosynthetic enzyme iPLA2, causing a decline in intracellular lysophospholipids that results in down-regulation of flagellin expression by *Salmonella*. These findings reveal a previously unrecognized immune-modulating regulatory cross-talk between endosomal TLR signaling and cytosolic NLR activation with significant implications for the establishment of infection with *Salmonella*.

Salmonella | inflammasome | flagellin | type 1 interferon | NLRC4

The innate immune system senses microbial pathogens through recognition of conserved entities collectively referred to as pathogen/microbe-associated molecular patterns (PAMPs/MAMPs). These entities interact with conserved pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), Nod-like receptors (NLRs), retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), and C-type lectin receptors (CLRs) that are expressed by immune cells and other cell types. Activation of PRRs by PAMPs is dictated by the availability and expression levels of PAMPs at different stages of infection and results in host responses which are vital for inflammation and immunity against pathogens (1). However, some pathogens, including *Salmonella* spp., a facultative intracellular pathogen, have evolved the ability to use these host responses for their own replication and establishment of infection (2).

Flagellin, the monomeric protein constituting bacterial flagella, is one of the key *Salmonella* effector molecules which binds and activates membrane-bound TLR-5 as well as the cytosolic sensor NLRC4 and plays a major role in generating inflammatory responses (3–5). In macrophages, flagellin as well as the rod protein PrgJ, which are inadvertently released into the host cytosol by the type III secretion system (T3SS), are detected by the NAIPs. In mice, seven NAIPs are present of which NAIP1 senses the T3SS needle protein, NAIP2 detects the T3SS inner rod protein, and NAIP5 and NAIP6 recognize flagellin (6–9). Humans however encode a single functional NAIP which has been recently shown to broadly detect multiple T3SS proteins and flagellin (10). Ligand binding to the NAIPs leads to recruitment and oligomerization of NLRC4 (11, 12). Activation of the NAIP-NLRC4 inflammasome by these effectors and activation of the NLRP3 inflammasome by an as yet unidentified aconitase-regulated *Salmonella* effector (13–15) results in caspase-1-dependent pyroptosis and production of active IL-1β which promotes clearance of the bacterium and protects the host against *Salmonella* (13, 16, 17). It is believed that as infection progresses, *Salmonella* circumvents this host-protective response by suppressing the expression of flagellin to lower than the resting levels usually expressed by bacteria in culture (18). Down-regulation of flagellin is essential for the bacterium to establish successful infection. Previous work has shown that a *Salmonella* Typhimurium strain modified to constitutively express flagellin (ST-FliC^{ON}) and therefore unable to naturally down-regulate flagellin expression is avirulent and cleared successfully from the host compared to its wild-type (WT) counterpart (17). Despite this central role of flagellin in *Salmonella* pathogenesis, the molecular mechanisms that regulate the physiological switch of *Salmonella* from a flagellin-high

Significance

While the effect of bacterial molecules on the host immune system is well studied, how host factors affect the expression of bacterial molecules is less appreciated. Here we uncover the impact of inflammasome activation and type 1 interferon on the expression of bacterial flagellin. Flagellin induces NLRC4 inflammasome-mediated pyroptosis causing clearance of *Salmonella*. We show that inflammasome activation also produces lysophospholipids which increase flagellin expression by *Salmonella* early in infection. We further demonstrate that as infection progresses, type 1 IFN inhibits NLRC4 and lysophospholipid synthesis, resulting in down-regulation of flagellin expression, a phenotype that the pathogen switches to during establishment of in vivo infection. These findings unravel pathways for biphasic regulation of expression of flagellin, a key *Salmonella* effector.

Author contributions: A.S.A., A.Q., and N.S. designed research; A.S.A., S.M.A., B.S.L., N.D., and N.S. performed research; K.T.H. contributed new reagents/analytic tools; A.S.A., S.M.A., B.S.L., N.D., A.Q., and N.S. analyzed data; and A.S.A., A.Q., and N.S. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

[1]Present address: Department of Medicine, University of Colorado Anschutz Medical Campus, Aurora, CO 80045.

[2]To whom correspondence may be addressed. Email: ayub@nii.ac.in or nsubrama@systemsbiology.org.

This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2002747117/-/DCSupplemental.

to a flagellin-low phenotype and aid in establishment of an intracellular niche within macrophages in vivo are incompletely understood.

Upon entry into macrophages, *Salmonella* resides in a vacuole called the *Salmonella*-containing vacuole (SCV) where it shuts down expression of the *Salmonella* pathogenicity island 1 (SPI-1) and concomitantly switches on expression of *Salmonella* pathogenicity island 2 (SPI-2), which is activated by the PhoP/PhoQ two-component system (19) and encodes genes required for intracellular replication. Prior work has shown that shutdown of SPI-1 in growth media that mimic conditions associated with the SCV such as acidic pH and low Mg^{2+} is also accompanied by repression of flagellin (20, 21). This is because low pH and low Mg^{2+} activate the PhoP/PhoQ system (20, 22, 23) and activated PhoP is believed to suppress expression of flagellin (21). A noteworthy issue relating to these early studies is that effects on PhoP/PhoQ-regulated genes were examined only during in vitro culture of bacteria in growth medium and not in the context of *S*. Typhimurium residence within macrophages. Therefore, the physiological contribution of these mechanisms to flagellin repression of intracellular *Salmonella* remains debatable. For example, contrary studies have shown that the effect of low pH on flagellin protein expression is observed only at a very low pH (pH = 3) and not at pH 5 (20) which is close to the physiologically relevant pH of the SCV (24, 25). Likewise, neither variation of extracellular Mg^{2+} nor reduced Mg^{2+} in the SCV was found to play a role in PhoP activation by *Salmonella* inside macrophages (26). Consequently, the regulatory mechanisms conventionally thought to repress flagellin expression by *Salmonella* remain controversial and there is scarce evidence to suggest that these factors are responsible for down-regulation of flagellin by bacteria residing within macrophages. Moreover, the physiological mechanisms that regulate repression of flagellin in vivo are unknown.

In this study we describe a host innate immune circuit that regulates expression of *Salmonella* flagellin during both the early/extracellular and the later/intracellular phases of macrophage infection with this pathogen. We find that during early infection of macrophages with *S*. Typhimurium, rapid NLRC4 inflammasome-dependent macrophage pyroptosis is necessary and sufficient for releasing a host lysophospholipid stimulus that promotes synthesis and release of flagellin from *Salmonella*. Unexpectedly, these host factors regulate not only the initial increase in flagellin production but also the later down-regulation of flagellin by *Salmonella* inside macrophages. This later effect is mediated by a natural type 1 IFN-dependent host negative feedback response that represses expression of NLRC4 and the lysophospholipid biosynthetic enzyme calcium-independent phospholipase A2 (iPLA2) within cells, causing a decline in intracellular lysophospholipids over time, which promotes eventual down-regulation of flagellin by intracellular bacteria. Our data identify host NLRC4 inflammasome activity as a temporal and biphasic regulator of expression of its own bacterial ligand, flagellin. We also describe a physiologically relevant type 1 IFN-mediated host mechanism that controls switching of *Salmonella* from a flagellin-high to a flagellin-low phenotype within macrophages in vivo. These findings have important implications for understanding the intricate evolutionary adaptations that shape host–pathogen cross-talk.

Results

IFNAR-Dependent Gradual Down-Regulation of Flagellin by Intracellular *Salmonella*. To understand how flagellin expression might be regulated during infection with *Salmonella*, we assessed time-dependent expression of flagellin by intracellular *Salmonella* in a gentamicin protection assay. Unless specified otherwise, experiments were done using the *S*. Typhimurium SL1344 strain. Ex vivo differentiated murine WT bone marrow-derived macrophages (BMDMs) were infected with log phase *S*. Typhimurium and then maintained in 100 μg/mL gentamicin, a bactericidal concentration that kills extracellular bacteria and blocks any possible reinfection of surviving BMDMs. Cells were lysed at different time points (6, 24, and 48 h) and intracellular bacteria were harvested and subjected to immunoblot or gene expression analysis. DnaK served as a control. The results showed that flagellin protein expression by intracellular bacteria declines gradually starting at 24 h with a considerable reduction by 48 h postinfection (Fig. 1 *A*, *Left*). This was accompanied by a reduction in expression of *fliC*, the gene encoding flagellin (Fig. 1 *A*, *Right*), suggesting that intracellular residence of *Salmonella* is associated with a gradual transcriptional repression of flagellin. These data are consistent with previous work showing the presence of motile, flagellated *Salmonella* inside macrophages 4 to 6 h after infection (27), and indicate that *Salmonella* does not lose expression of flagellin immediately upon internalization by macrophages but rather displays a progressive loss of flagellin expression over time.

It has been reported that type 1 IFN signaling dampens inflammasome activation (28). Increased ASC specks, caspase-1 activation, and IL-1β production are observed in IFNAR-deficient macrophages infected with *Salmonella* (29). Given that flagellin is one of the main triggers of inflammasome activation by *Salmonella* (4), we asked whether sustenance of flagellin expression due to lack of IFNAR signaling may be one of the reasons for increased inflammasome activity in $IFNAR^{-/-}$ cells. BMDMs conditioned with 0.22-μm filter-sterilized supernatants from infected cells showed greater down-regulation of flagellin by intracellular bacteria with nearly complete abrogation by 48 h when compared to BMDMs conditioned with cell supernatant from uninfected cells (Fig. 1*B*), suggesting that a soluble factor released by infected cells promotes down-regulation of flagellin by intracellular bacteria. To test if this factor may be type 1 IFN, we infected WT or $IFNAR^{-/-}$ BMDMs with *S*. Typhimurium and analyzed expression of flagellin. Compared to intracellular bacteria recovered from WT BMDMs which down-regulated flagellin, bacteria isolated from $IFNAR^{-/-}$ BMDMs showed a significant reversal of flagellin down-regulation both at the mRNA (Fig. 1 *C*, *Right*) and the protein (Fig. 1 *C*, *Left*) level. Both WT and $IFNAR^{-/-}$ BMDMs showed equivalent induction of IFN-β (*SI Appendix*, Fig. S3*D*). We also examined the effect of IFNAR signaling on flagellin down-regulation by *Salmonella* in situ. WT and $IFNAR^{-/-}$ BMDMs were infected with a reporter strain of *S*. Typhimurium 14028 in which the *fliC* promoter drives expression of GFP (*S*. Typhimurium 14028 *fliC*-GFP), and intracellular bacteria were visualized by imaging at 6, 24, and 48 h after infection. Consistent with our population-level immunoblot data, a decline in *fliC* expression was also seen at the level of single bacteria and single cells in WT BMDMs (Fig. 1*D*). This was reversed in $IFNAR^{-/-}$ BMDMs (Fig. 1*D*), confirming that IFNAR signaling promotes down-regulation of flagellin by intracellular bacteria. Importantly, similar results were obtained during in vivo infection. WT and $IFNAR^{-/-}$ mice were infected intraperitoneally (i.p.) with *S*. Typhimurium, and expression of flagellin by intracellular bacteria recovered from splenic monocytes at days 2 and 5 postinfection was analyzed. The results showed that bacteria recovered from WT spleens down-regulated flagellin by day 5. However, bacteria recovered from $IFNAR^{-/-}$ spleens showed minimal down-regulation of flagellin, suggesting that IFNAR signaling is required for *Salmonella* to switch from a flagellin-high to a flagellin-low state in vivo (Fig. 1*E*).

NLRC4 Inflammasome-Dependent Pyroptosis Promotes Early Increase in Flagellin Production by *Salmonella*. To understand the mechanism of down-regulation of flagellin expression by intracellular *Salmonella*, we reasoned that an intracellular stimulus maintains flagellin expression, and IFNAR-mediated depletion of that stimulus might cause time-dependent down-regulation of flagellin expression by intracellular *Salmonella*. To investigate the nature of the stimulus, we infected macrophages briefly with WT *S*. Typhimurium

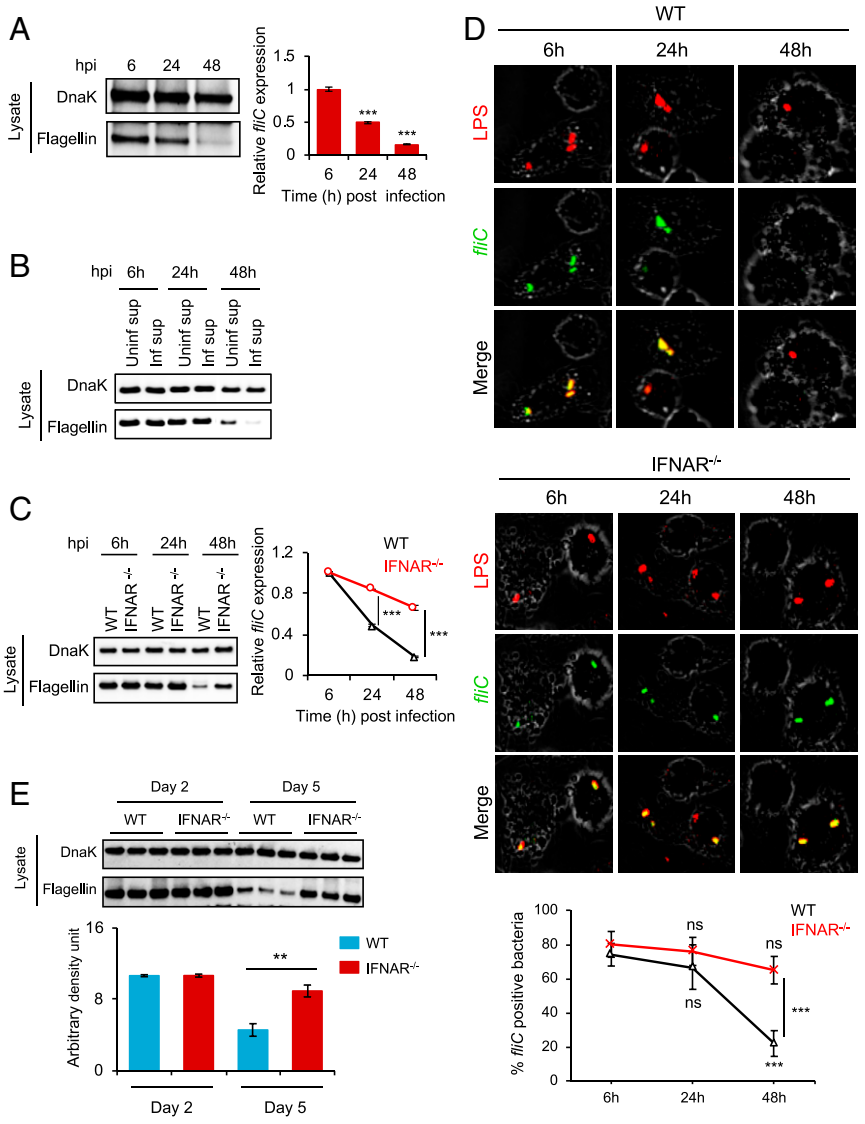


Fig. 1. IFNAR signaling promotes down-regulation of flagellin by *Salmonella*. (*A*) Immunoblot (*Left*) and qRT-PCR (*Right*) for flagellin expression by intracellular *Salmonella* obtained from infected mCSF-differentiated BMDMs at the indicated times. WT BMDMs were infected with 25 MOI of log phase *S.* Typhimurium for 30 min. Extracellular bacteria were removed and cells were incubated in complete medium containing gentamicin (100 μg/mL) for 6, 24, and 48 h. At each time point, intracellular bacteria were harvested. DnaK was used as a loading control for immunoblots and as a housekeeping gene to which *fliC* expression was normalized in qPCR. *fliC* gene expression at 6 h was set to 1. Hpi, hours postinfection. (*B*) Immunoblot for flagellin and DnaK showing that a soluble factor from infected cells promotes down-regulation of flagellin by intracellular *Salmonella.* Cell-free supernatant from WT BMDMs uninfected or infected with log phase *S.* Typhimurium (25 MOI) was used to condition fresh BMDMs for 5 h prior to infection of these cells with *S.* Typhimurium. Intracellular bacteria were harvested at the indicated times and expression of flagellin and DnaK was analyzed by immunoblot. (*C*) Immunoblot (*Left*) and qPCR (*Right*) for flagellin expression by intracellular *S.* Typhimurium obtained from infected WT and IFNAR$^{-/-}$ BMDMs (25 MOI) at the indicated times postinfection. Expression of *fliC* was normalized to *dnaK*. (*D*) Representative confocal immunofluorescence images (*Top* and *Center*) and quantification (*Bottom*) showing *fliC* expression by *S.* Typhimurium in situ. WT and IFNAR$^{-/-}$ BMDMs were infected with log phase *fliC*-GFP reporter strain of *S.* Typhimurium 14028 (25 MOI or 30 min) followed by gentamicin protection (100 μg/mL) for the indicated times. Cells were stained with antibody to *S.* Typhimurium LPS. Intracellular *Salmonella* were visualized as LPS-positive (red), flagellin-positive (green), and double positive (yellow). The percentage of *fliC*-positive bacteria per condition was enumerated as a fraction of total LPS-positive bacteria. At least 25 cells were analyzed per condition. (*E*) Immunoblot (*Top*) and corresponding densitometric quantification (*Bottom*) for flagellin and DnaK expression by intracellular *S.* Typhimurium obtained from spleens of infected WT and IFNAR$^{-/-}$ mice (200 colony forming unit [CFU] i.p.). Intracellular *Salmonella* were harvested from adherence-purified monocytes on days 2 and 5 postinfection, and protein expression was analyzed by immunoblotting. Band intensities of flagellin were normalized to DnaK using ImageJ software. Data are representative of three (*A*–*C*) or two (*D* and *E*) independent experiments. Error bars on graphs are mean ± SD (*A*, *C*, and *E*) or mean ± SEM (*D*). $^{**}P < 0.01$, $^{***}P < 0.001$. ns, not significant.

(KK1004; *SI Appendix*, Table S1) (50 multiplicity of infection [MOI] for 30 min; this MOI was chosen so as to liberate sufficient amounts of the intracellular host stimulus into the supernatant) and analyzed secretion of flagellin in the supernatants of *Salmonella*–macrophage cocultures. We measured flagellin released in the supernatant because at this early stage of infection, flagellin is contributed predominantly by extracellular *Salmonella* present in the culture medium (we refer to this as flagellin release by extracellular *Salmonella*) and translation of flagellin in *Salmonella* is coupled to its secretion (30). Flagellin was readily detected in the supernatants derived from bacteria–macrophage coculture at levels much higher than those obtained from *Salmonella* grown in cell culture medium without macrophages. Similar amounts of flagellin were detected regardless of whether macrophages were infected with WT *S.* Typhimurium KK1004 or a *fliD* mutant strain that expresses flagellin but lacks the ability to polymerize it into filaments and therefore does not express surface flagella (*S.* Typhimurium KK1004 *fliD*:Tn10; Fig. 2 *A*, *Left*). This suggests that early in infection, interaction with macrophages triggers release of flagellin from *Salmonella*. Moreover, the secreted flagellin is monomeric in nature and not the result of degradation or depolymerization of surface flagella.

Macrophages undergo rapid caspase-1-dependent pyroptotic cell death upon infection with *Salmonella* which is primarily dependent on activation of the NLRC4 inflammasome by flagellin (13, 31) (Fig. 2 *A*, *Right*). Therefore, we examined if there was any relationship between infection-induced pyroptosis and release of flagellin by the bacterium. Inhibition of cell death with zVADfmk (a pan-caspase inhibitor) or zYVAD (a caspase-1-specific inhibitor) during *S.* Typhimurium infection of macrophages almost completely abrogated flagellin release from the pathogen (Fig. 2*B*). Consistent with these findings, infection of NLRC4$^{-/-}$ or Caspase-1/11$^{-/-}$ but not NLRP3$^{-/-}$ macrophages with *S.* Typhimurium induced release of markedly lower amounts of flagellin from the pathogen compared to WT macrophages (Fig. 2*B*). These results suggest that sensing of a macrophage pyroptosis-derived stimulus triggers secretion of flagellin from *Salmonella*. Filter-sterilized supernatants from *S.* Typhimurium-infected WT macrophages led to increased β-galactosidase activity in a *S.* Typhimurium KK1004 reporter

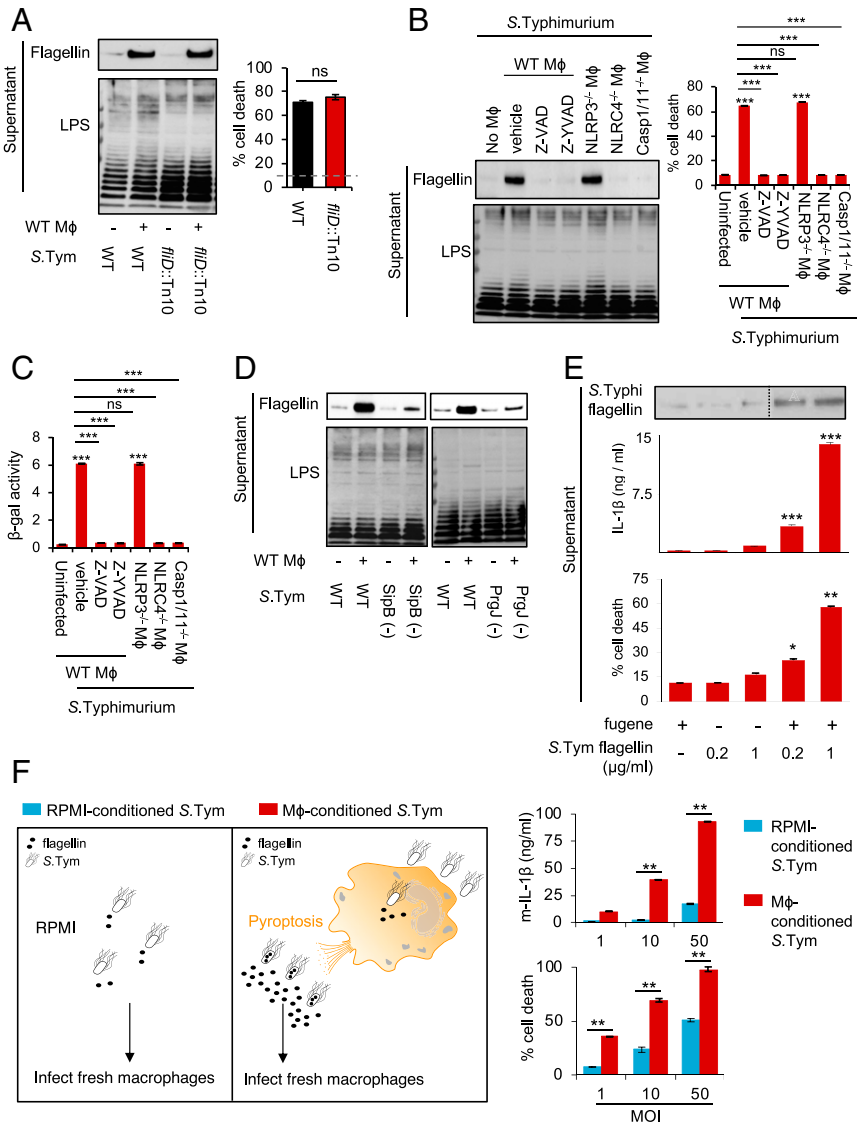


Fig. 2. NLRC4 inflammasome-mediated pyroptosis promotes increase in flagellin production by *Salmonella*. (*A*) Immunoblot for flagellin and LPS (*Left*) and assay for LDH (*Right*) in filter-sterilized supernatants of peritoneal macrophages infected with log phase WT *S*. Typhimurium KK1004 or its congenic aflagellar mutant (*fliD*::Tn10) (50 MOI for 1 h). Flagellin and LPS released by bacteria were analyzed by immunoblot and macrophage cell death was assessed by spectrophotometric detection of LDH. (*B*) Immunoblot for flagellin and LPS (*Left*) and assay for LDH (*Right*) in filter-sterilized supernatants of peritoneal macrophages of the indicated genotypes infected with log phase *S*. Typhimurium (50 MOI for 1 h). WT macrophages were infected in presence or absence of the pan-caspase inhibitor zVADfmk (100 μM) and caspase-1-specific inhibitor zYVADfmk (100 μM). (*C*) β-Galactosidase (β-gal) reporter assay for transcriptional induction of flagellin (*fliC*) in response to supernatants from macrophages treated as in *B*. *S*. Typhimurium KK1004 with the *fliC* promoter region fused to a promoterless lacZ (*S*.Tym *fliC-lacZ*; KK1110) was treated with filter-sterilized supernatants from macrophages in *B* (horizontal axes) and β-gal activity was determined colorimetrically. (*D*) Immunoblot for flagellin and LPS in supernatants of macrophages infected with log phase WT *S*. Typhimurium SL1344 or its SipB-deficient derivative and WT *S*. Typhimurium KK1004 or its PrgJ-deficient derivative for 1 h. (*E*) Immunoblot for flagellin released by *S*. Typhi upon treatment with supernatants from macrophages transfected with *S*. Typhimurium flagellin. LPS-primed peritoneal macrophages were transfected with 200 ng or 1,000 ng *S*. Typhimurium flagellin for 3 h. Cell-free supernatants were analyzed for IL-1β and LDH (*Middle* and *Lower*), and their ability to trigger release of flagellin from *S*. Typhi using monoclonal antibodies specific to *S*. Typhi flagellin (*Upper*). (*F*, *Left*) Schematic of the experimental setup. (*F*, *Right*) Assay for IL-1β (*Top*) and LDH (*Bottom*) released from macrophages upon infection with *S*. Typhimurium previously exposed to peritoneal macrophages or plain cell culture medium. Log phase *S*. Typhimurium was either exposed to cell culture medium (RPMI) or peritoneal macrophages for 1 h at 37 °C. Bacteria were washed and used to infect LPS-primed macrophages at the indicated MOI for 1 h. Cell-free supernatants were analyzed for IL-1β and LDH. Data in *A*–*F* are representative of three independent experiments. Error bars are mean ± SD of triplicates. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. ns, not significant.

strain carrying a promoterless *Lac* operon fused to the *fliC* promoter (*S*.Tym *fliC-lacZ*; KK1110). This increased reporter activity was not observed with supernatants from pyroptosis-defective macrophages, suggesting that a stimulus released upon macrophage pyroptosis activates de novo transcription from the *fliC* promoter (Fig. 2*C*). Furthermore, SipB-deficient *S*. Typhimurium, or PrgJ-deficient *S*. Typhimurium which do not efficiently induce caspase-1-dependent pyroptosis (32, 33) (*SI Appendix*, Fig. S1*A*) produced substantially lower amounts of flagellin upon coculture with macrophages compared to their respective parent WT *S*. Typhimurium strain (Fig. 2*D*). The stimulatory effect of pyroptosis was specific to flagellin as there was no difference in the amount of LPS present in supernatants of macrophage–*Salmonella* cocultures irrespective of whether SipB-deficient, PrgJ-deficient or their respective congenic parental WT *S*. Typhimurium strain was used to infect macrophages (Fig. 2*D*). Together, these results suggest that NLRC4 and caspase-1-dependent pyroptosis of macrophages liberates a stimulus which activates transcription and release of flagellin from *Salmonella*. Furthermore, NLRP3 is not involved in early pyroptosis upon *Salmonella* infection (31) and the consequent release of flagellin from the pathogen (Fig. 2 *B* and *C*).

In macrophages, the NLRC4 inflammasome is activated by flagellin and the rod protein PrgJ (33). We therefore asked if inflammasome activation by flagellin alone may be sufficient for further enhancing production of flagellin from bacteria in a feedforward, autoamplifying circuit. To test this, we infected macrophages with either WT or flagellin-deficient *S*. Typhimurium and examined the ability of filter-sterilized culture supernatants from these cocultures to trigger flagellin from *S*. Typhi. A highly specific anti-*S*. Typhi flagellin monoclonal antibody enabled us to discriminate flagellin molecules derived from these two closely related *Salmonella* species (34). The results showed that flagellin release from *S*. Typhi was much less when it was incubated with supernatant from macrophages infected with aflagellar *S*. Typhimurium (*S*. Typhimurium KK1004 *flhD::Tn10*, which activates caspase-1 poorly) (4), even at a MOI 10 times higher than of WT *S*. Typhimurium KK1004. There was however no difference in the amount of LPS present in *S*. Typhi supernatants under these conditions (*SI Appendix*, Fig. S1 *B*, *Left*). As expected,

macrophages infected with WT *S*. Typhimurium KK1004 produced greater IL-1β compared to those infected with its aflagellar counterpart and the presence of IL-1β correlated with the presence of a flagellin-inducing stimulus in these supernatants (*SI Appendix*, Fig. S1 *B*, *Right*). These results suggest that flagellin-dependent inflammasome activation is sufficient to generate a host stimulus capable of activating flagellin production from *Salmonella*. To confirm this, we delivered *S*. Typhimurium flagellin (endotoxin-free, ultrapure) intracellularly into LPS-primed macrophages and analyzed cell supernatants for IL-1β, lactate dehydrogenase (LDH) and their ability to activate release of flagellin from *S*. Typhi. As expected, cells transfected with flagellin showed IL-1β and LDH release in a dose-dependent fashion (Fig. 2 *E*, *Middle* and *Bottom*). More importantly, supernatants derived from these cells also triggered flagellin release from *S*. Typhi (Fig. 2 *E*, *Top*). Taken together, these results demonstrate that NLRC4 inflammasome-dependent pyroptosis following intracellular delivery of flagellin is sufficient to liberate a host stimulus that can induce secretion of flagellin from pathogenic *Salmonella*.

We next asked if increased flagellin expression by bacteria exposed to macrophages may confer on them an increased ability to induce pyroptosis in healthy cells. To test this, we infected macrophages with *S*. Typhimurium which was previously either incubated with RPMI or cocultured with macrophages and measured LDH and IL-1β release in the supernatant. Compared to bacteria that had not been previously cocultured with macrophages, *Salmonella* previously exposed to macrophages had an increased ability to trigger cell death and IL-1β secretion in fresh macrophages (Fig. 2*F*). These results indicate that early interaction of *Salmonella* with macrophages leads to generation of bacteria with higher pyroptotic capacity, a modulation that is reported to promote clearance of the pathogen from the system (17) (*SI Appendix*, Fig. S9 *A* and *B*).

Lysophospholipids from Pyroptotic Macrophages Enhance Flagellin Production by *Salmonella*. We next examined the identity of the flagellin-inducing host stimulus released upon macrophage pyroptosis. The ability of supernatants derived from infected macrophages to trigger flagellin release from *S*. Typhi was not affected by Proteinase K digestion, indicating that the stimulus was nonproteinaceous in nature (*SI Appendix*, Fig. S1*C*). However, delipidification of supernatant from *S*. Typhimurium-infected macrophages using chloroform–methanol extraction abrogated its ability to trigger flagellin release from *S*. Typhi (Fig. 3*A*), indicating that the stimulus was lipid in nature. We have previously shown that lysophosphatidylcholine (LPC) produced by intestinal epithelial cells triggers release of monomeric flagellin from *Salmonella* (35). To test if lysophospholipids are also released by pyroptotic macrophages and may be the active components in macrophage supernatants, we infected RAW 264.7 macrophage cells with *S*. Typhimurium and analyzed culture supernatants for the presence of lysophospholipids by thin layer chromatography (TLC) and mass spectrometry, as well as for their ability to trigger release of flagellin from *S*. Typhi. The results showed that supernatant from infected macrophages could readily induce release of flagellin from *S*. Typhi (*SI Appendix*, Fig. S1*D*). TLC analysis revealed that the supernatant contained LPC as one of its lipid components (Fig. 3*B*), which was confirmed by liquid chromatography-mass spectrometry (LC-MS) (*SI Appendix*, Fig. S1*E*). Inhibition of the lysophospholipid biosynthetic enzyme, iPLA2, but not calcium-sensitive cytosolic PLA2 (cPLA2) in macrophages caused a significant reduction in cellular levels of LPC and in the amounts of LPC released upon infection with *Salmonella* (Fig. 3*C*), without any effect on macrophage cell death as measured by LDH release in the supernatant (Fig. 3 *D*, *Right*). This reduction correlated with reduced flagellin release from *Salmonella* cultured with macrophages in presence of the iPLA2 inhibitor (Fig. 3 *D*, *Left*). The levels of extracellular LPC and consequently flagellin release from *Salmonella* were also reduced if cell death was inhibited with zVADfmk or zYVAD or infections were performed with macrophages lacking NLRC4 or Caspase-1/11, which do not undergo early cell death with *Salmonella* (Figs. 2*B* and 3*E*). In contrast, cells lacking NLRP3 readily released LPC upon infection with *Salmonella* and consequently induced release of flagellin from bacteria (Figs. 2 *B*, *Left* and 3*E*). These results indicate that lysophospholipids including LPC produced by iPLA2 and released upon pyroptosis of *Salmonella*-infected macrophages activate release of flagellin from this pathogen. As expected, purified lysophospholipids triggered release of flagellin from *Salmonella*. Moreover, SipB-deficient or PrgJ-deficient bacteria were as competent as their respective parental WT *S*. Typhimurium strain at producing flagellin in response to lysophospholipids (*SI Appendix*, Fig. S1*F*), confirming that reduced flagellin secretion by SipB-deficient or PrgJ-deficient *Salmonella* (Fig. 2*D*) was because of reduced availability of lysophospholipids due to inefficient induction of macrophage pyroptosis by these bacteria (*SI Appendix*, Fig. S1*A*) (32, 33) and not due to an intrinsic defect in flagellin secretion or secondary effects of SipB/PrgJ deficiency.

Cytosolic LPS activates Caspase-11 (36, 37). To determine if Caspase-11 is involved in modulating release of flagellin by *Salmonella*, we infected WT and Caspase-11$^{-/-}$ macrophages with *Salmonella* and analyzed release of extracellular flagellin. Infection-induced caspase-1 activation (*SI Appendix*, Fig. S1*G*), cell death (Fig. 3 *F*, *Right*), and release of flagellin by *Salmonella* (Fig. 3 *F*, *Left*) were not affected by Caspase-11 deficiency. Accordingly, WT and Caspase-11$^{-/-}$ macrophages released similar amounts of LPC into the supernatant upon infection (Fig. 3*G*). Together these results indicate that release of intracellular LPC and consequent release of flagellin by *Salmonella* is dependent on pyroptosis mediated by NLRC4 and Caspase-1 and independent of Caspase-11.

We next investigated the mechanism by which LPC regulates flagellin expression in *Salmonella*. We have previously shown that LPC can increase transcription of flagellin from the *fliC* promoter (35). More recently, LPC was also found to promote production of *Salmonella* invasion-promoting molecules, SipA and SipC, through sustained induction of the SPI-1 transcriptional regulator, *hilA* (38), which raised the possibility that LPC sensing by *Salmonella* might modulate a regulator common to the SPI-1 and flagellar regulons. The DNA binding protein HilD is a dominant regulator of *hilA* transcription (39) and also directly activates transcription of the flagellar master operon *flhDC* (40). We therefore analyzed HilD protein levels and *flhDC* transcription in *Salmonella* using FLAG-tagged HilD and *flhDC*-luciferase reporter strains respectively of *S*. Typhimurium 14028. Stimulation with LPC led to a marked increase in HilD amounts (*SI Appendix*, Fig. S1*H*), which was accompanied by an increase in *flhDC* transcription (*SI Appendix*, Fig. S1*I*). These results suggest that LPC enhances flagellin expression in a HilD-*flhDC*-dependent manner.

Temporal Down-Regulation of NLRC4 and iPLA2 Expression in *Salmonella*-Infected Macrophages. Our data (Fig. 1*A*) and those of others (21) suggest that *Salmonella* eventually switches to a flagellin-low phenotype inside macrophages. We reasoned that the feedforward circuit that amplifies flagellin production in response to macrophage pyroptosis and lysophospholipids, as described in Figs. 2 and 3, must be arrested for this to occur. To test this, we analyzed expression of iPLA2 and inflammasome components at varying times post *S*. Typhimurium infection of WT BMDMs. The results showed that NLRC4 and iPLA2 protein expression was substantially down-regulated to subbaseline levels post *Salmonella* infection (Fig. 4*A*). The decrease in NLRC4 and iPLA2 expression was also seen at the mRNA level in BMDMs (Fig. 4*B*) and in vivo in adherence-purified monocytes from spleens of *S*. Typhimurium-infected mice (Fig. 4*C*). In contrast, the expression of NLRP3, ASC, Caspase-1, and cPLA2

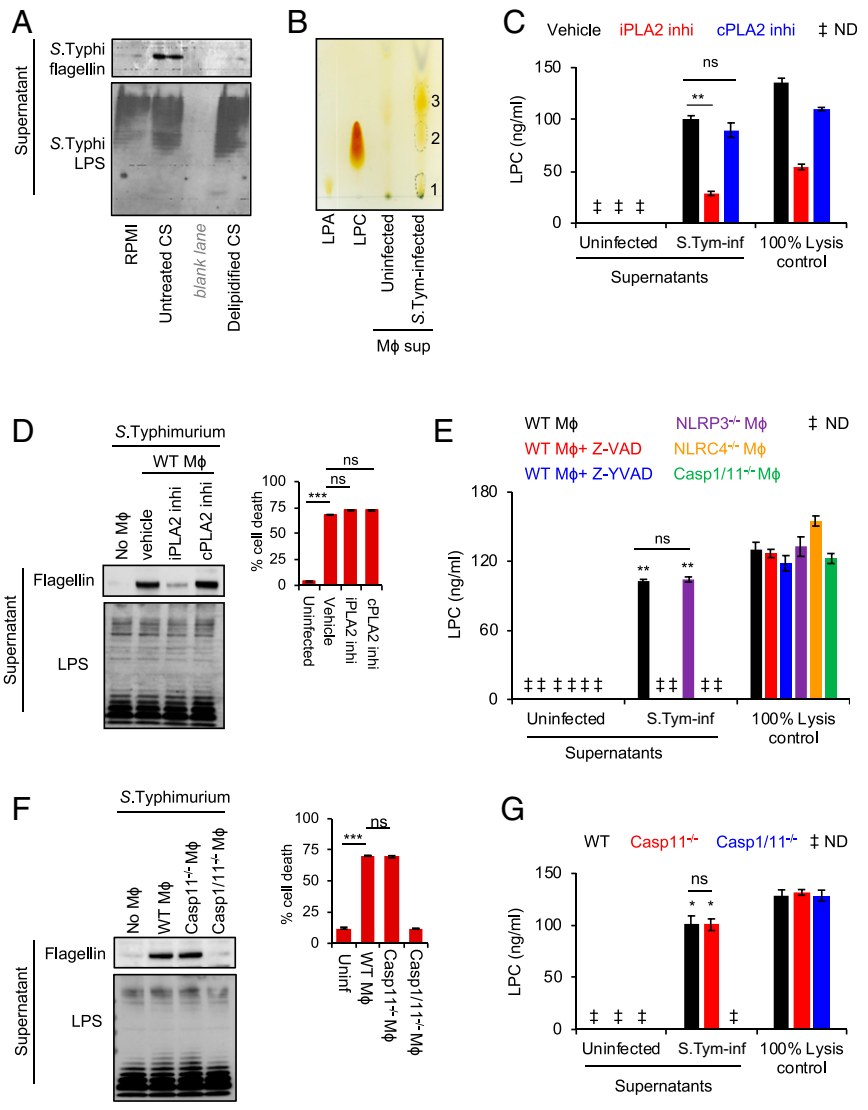


Fig. 3. Pyroptosis-derived lysophospholipids amplify production of flagellin by *Salmonella*. (*A*) Immunoblot for flagellin and LPS released by *S*. Typhi in response to delipidified supernatant from *S*. Typhimurium-infected macrophages. Serum-free cell culture supernatant from log phase *S*. Typhimurium-infected macrophages (50 MOI) was extracted with chloroform/methanol (1:1). The aqueous phase was incubated with *S*. Typhi. (*B*) Analysis of lipids in supernatant of *S*. Typhimurium-infected macrophages by TLC. Cell-free supernatant from RAW 264.7 cells either left uninfected or cocultured with log phase *S*. Typhimurium (50 MOI) was extracted with chloroform/methanol (1:1) and subjected to TLC. Lipids were visualized with iodine. LPA, purified LPA (lysophosphatidic acid); LPC, purified LPC (lysophosphatidylcholine). (*C*) Estimation of LPC released by infected macrophages upon PLA2 inhibition. Peritoneal macrophages from WT mice were treated with vehicle (dimethylsulfoxide [DMSO]) or with inhibitors specific for iPLA2 (FKGK-11, 30 μM) or cPLA2 (pyrrophenone, 1 μM) for 12 h. Cells were infected with 50 MOI log phase *S*. Typhimurium for 1 h and supernatants were collected. For 100% lysis control, uninfected cells were lysed in equal volume of lysis buffer. LPC in infected cell supernatants and the 100% lysis control was estimated by ELISA. (*D*) Immunoblot for flagellin and LPS (*Left*), and assay for LDH (*Right*) in filter-sterilized supernatants of macrophages infected with *S*. Typhimurium. Peritoneal macrophages from WT mice were treated with vehicle or with inhibitors specific for iPLA2 or cPLA2 as described in *C*, followed by infection with 50 MOI log phase *S*. Typhimurium for 1 h. (*E*) Estimation of LPC in supernatants of peritoneal macrophages of the indicated genotypes infected with 50 MOI log phase *S*. Typhimurium for 1 h. WT macrophages were infected in presence or absence of the pan-caspase inhibitor zVADfmk (100 μM) and caspase-1-specific inhibitor zYVADfmk (100 μM). LPC in infected cell supernatants and the 100% lysis control was quantified by ELISA. (*F*) Immunoblot for flagellin and LPS (*Left*) and LDH assay for macrophage cell death (*Right*) in filter-sterilized supernatants of WT, Caspase-11$^{-/-}$, and Caspase-1/11$^{-/-}$ peritoneal macrophages infected with 50 MOI log phase *S*. Typhimurium for 1 h. (*G*) Estimation of LPC in supernatants of WT, Caspase-11$^{-/-}$, and Caspase-1/11$^{-/-}$ peritoneal macrophages infected with *S*. Typhimurium as in *F*. LPC in the supernatants and the 100% lysis control was estimated by ELISA. Data are representative of two independent experiments. Error bars are mean ± SD of triplicates. ND, not detected. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. ns, not significant.

did not decrease below their level of expression in uninfected cells, indicating that such down-regulation was seemingly specific to NLRC4 and iPLA2 (Fig. 4 *A*–*C*). These results indicate that *Salmonella* infection leads to decreased NLRC4 and iPLA2 expression in macrophages both in vitro and in vivo.

Because a significant proportion of *Salmonella*-infected macrophages undergo rapid NLRC4-dependent cell death (Fig. 2*B*), we wondered if the observed down-regulation of NLRC4 over time could simply be due to a preferential selection of preexisting NLRC4-low cells in the macrophage population which are resistant to cell death, rather than an active infection-induced repression of NLRC4. To test this, we analyzed gene expression in Caspase-1/11$^{-/-}$ and Gasdermin D$^{-/-}$ BMDMs which are refractory to *Salmonella*-induced pyroptosis (41, 42). Time-dependent down-regulation of NLRC4 and iPLA2 was also observed in infected Caspase-1/11$^{-/-}$ and Gasdermin D$^{-/-}$ BMDMs (*SI Appendix*, Fig. S2 *A* and *B*), indicating that repression of NLRC4 and iPLA2 expression is independent of macrophage cell death.

A Type 1 IFN-Dependent Host Response Fosters a NLRC4 and iPLA2-Low State in Macrophages. We next sought to investigate the mechanism by which NLRC4 and iPLA2 expression is down-regulated in *Salmonella*-infected macrophages. We first asked if reduction in NLRC4 and iPLA2 levels was due to an active targeting of these genes by the pathogen. We infected BMDMs either with viable *S*. Typhimurium or bacteria that had been rendered nonviable by exposure to heat or gentamicin and assessed expression of NLRC4, iPLA2, cPLA2, and other inflammasome components (Fig. 5*A* and *SI Appendix*, Fig. S3*A*). Killed bacteria were as efficient as live bacteria in promoting down-regulation of NLRC4 and iPLA2 (Fig. 5*A*). None of the other inflammasome components or cPLA2 were down-regulated (*SI Appendix*, Fig. S3*A*). Similar results were obtained with *Pseudomonas aeruginosa*, another flagellated Gram-negative bacterium (Fig. 5*A* and *SI Appendix*, Fig. S3*A*). These results suggest that suppression of NLRC4 and iPLA2 expression in macrophages does not require metabolically active or invasive bacteria and may be a natural host response to bacterial recognition by cell surface-associated or endosomal TLRs. To test this possibility, we treated

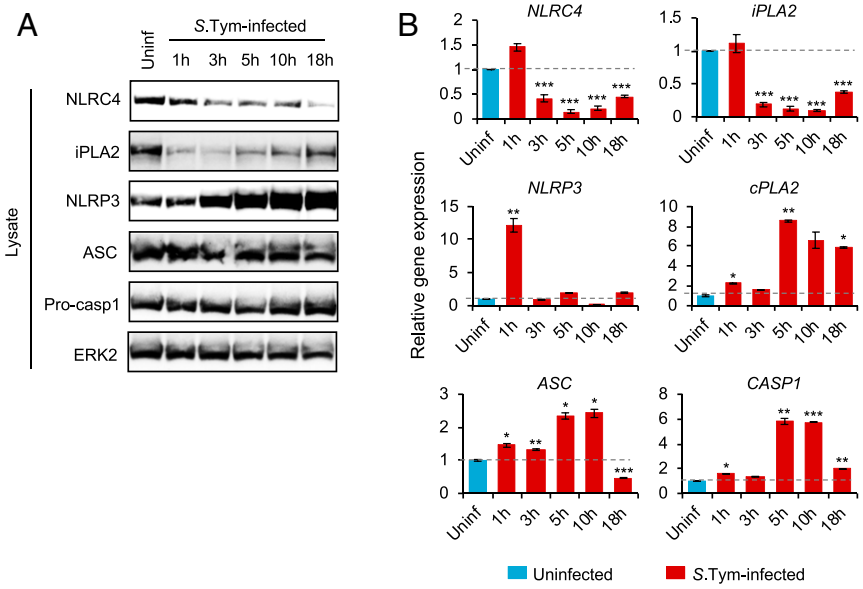


Fig. 4. Kinetics of NLRC4 and iPLA2 down-regulation by infected macrophages. (*A*) Immunoblot for inflammasome components and iPLA2 in lysates of uninfected BMDMs or BMDMs infected with log phase *S*. Typhimurium (25 MOI for 30 min), followed by gentamicin protection (100 μg/mL) for the indicated times. (*B*) qPCR for the indicated genes in *Salmonella*-infected BMDMs. WT BMDMs were infected as in *A*. Gene expression was normalized to β-actin. Gene expression in uninfected cells at each time point was set to 1 and is represented by the dashed line. Gene expression in *Salmonella*-infected cells is represented relative to the respective uninfected control at each time point (i.e., cells incubated in media alone for the same time). (*C*) qPCR for the indicated genes in splenocytes of *Salmonella*-infected mice. WT mice were uninfected or infected i.p. with 200 CFU log phase *S*. Typhimurium. On days 2, 3, and 5 postinfection, splenocytes were harvested and gene expression in adherence-purified $CD11b^+$ monocytes was analyzed. Gene expression in uninfected splenocytes was set to 1 and is denoted by the dashed line. Data are representative of three independent experiments (*A* and *B*) or two independent experiments with four mice per group (*C*). Error bars are mean ± SD of triplicates. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

BMDMs with purified TLR ligands and analyzed gene expression of NLRC4 and iPLA2. LPS and Poly I:C but not flagellin mimicked the effect of intact *Salmonella* in inducing shutdown of NLRC4 and iPLA2 expression in BMDMs (Fig. 5*B*). Expression of other genes was not reduced and as expected, expression of NLRP3 was up-regulated in response to LPS and flagellin, two TLR ligands that activate NF-κB (43) (*SI Appendix*, Fig. S3*B*).

We then assessed a requirement for TLR signaling adapters in the expression of these genes. Interestingly, TRIF deficiency but not MyD88 deficiency significantly rescued the decline in NLRC4 and iPLA2 expression in response to *S*. Typhimurium infection, suggesting that TRIF-dependent signaling is required for down-regulation of these genes (Fig. 5*C*). A key feature of TRIF engagement by TLRs is the production of type 1 IFN, primarily IFN-β in response to recognition of LPS during *Salmonella* infection (44). Purified IFN-β mimicked the effect of live *Salmonella* infection in down-regulating expression of NLRC4 and iPLA2 in macrophages (Fig. 5*D*). This effect was reversed if macrophages were pretreated with actinomycin D (an inhibitor of transcription) or cycloheximide (an inhibitor of translation), indicating that such down-regulation was dependent on IFN-β-induced fresh mRNA and protein synthesis (*SI Appendix*, Fig. S3*C*). Further, BMDMs lacking IFNAR, the activating receptor required for IFN-β signaling did not down-regulate NLRC4 or iPLA2 expression to subbaseline levels in response to *Salmonella* infection, rather these cells showed a modest early increase in expression of these genes (Fig. 5*E*). As expected, $IFNAR^{-/-}$ BMDMs were as competent as WT BMDMs in producing IFN-β but were deficient in inducing expression of the IFN-stimulated gene (ISG) MX2 (*SI Appendix*, Fig. S3*D*).

Finally, we assessed the role of soluble type 1 IFN released from infected cells in inducing a NLRC4- and iPLA2-low state in uninfected bystander cells. We conditioned WT and $IFNAR^{-/-}$ BMDMs with filter-sterilized supernatant from *S*. Typhimurium-infected macrophages and analyzed gene expression. Infected cell supernatant down-regulated expression of NLRC4 and iPLA2 in WT BMDMs and this was significantly reversed in $IFNAR^{-/-}$ BMDMs (Fig. 5*F*), confirming that soluble type 1 IFN produced by *Salmonella*-infected macrophages fosters a NLRC4- and iPLA2-low state in uninfected macrophages. The detailed mechanism by which type 1 IFN represses NLRC4 and iPLA2 expression is unclear at the moment. Diverse mechanisms can operate downstream of IFNAR, for example, up-regulation of transcriptional repressors and remodeling of chromatin (45), induction of proteins involved in mRNA degradation (46), and induction of suppressors such as SOCS-1 (47) and IL-10 (48). It

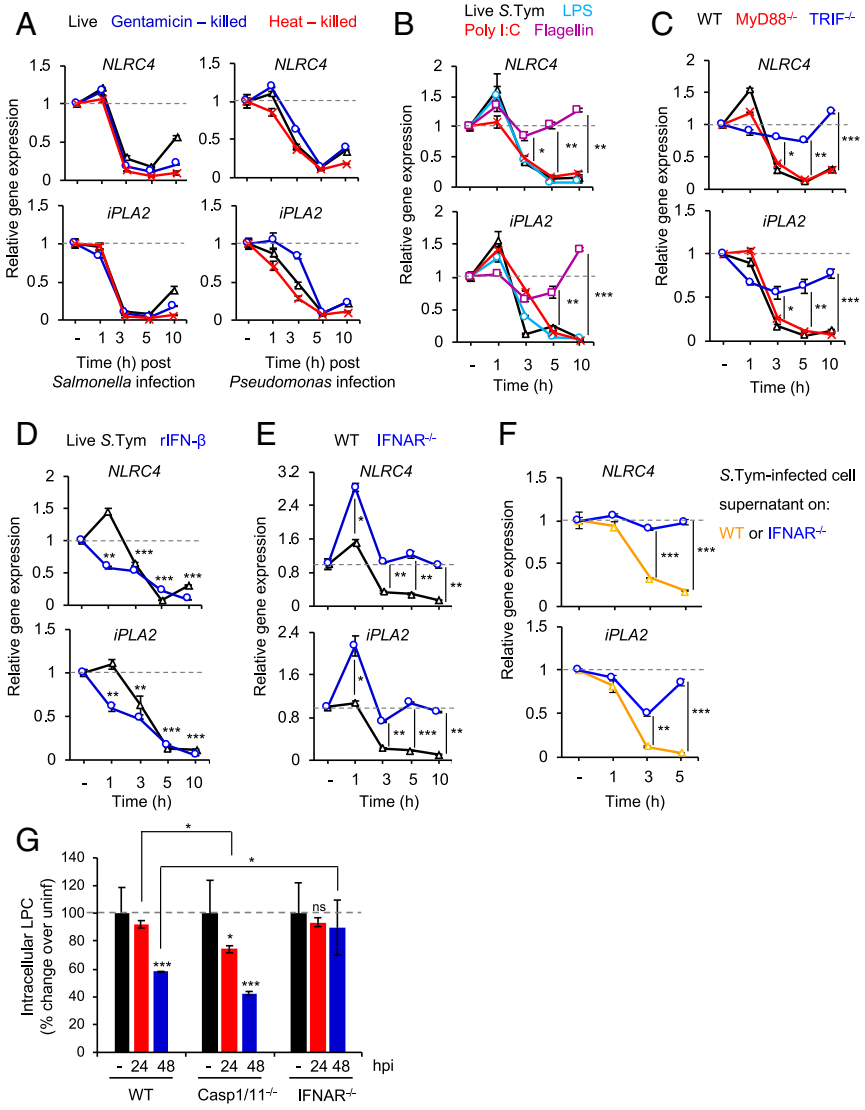


Fig. 5. Type 1 IFN signaling fosters a NLRC4-, iPLA2-, and LPC-low state in macrophages. (*A*) qPCR for NLRC4 and iPLA2 showing down-regulation of these genes in infected BMDMs over time. WT BMDMs were infected with 25 MOI of live, heat-killed (90 °C, 30 min) or gentamicin-killed (100 μg/mL, 30 min) *S.* Typhimurium or *P. aeruginosa* for 30 min. Infection was synchronized at 1,500 rpm for 5 min. Extracellular bacteria were removed followed by gentamicin protection for the indicated times. Gene expression was normalized to β-actin and the uninfected control at that time point (dashed line, set to 1) as described in Fig. 4*B*. (*B*) qPCR for NLRC4 and iPLA2 in BMDMs infected with log phase *S.* Typhimurium or treated with TLR ligands. WT BMDMs were infected as in *A* or treated with TLR agonists LPS (1 μg/mL), flagellin (1 μg/mL), or poly I:C (5 μg/mL) for the indicated times. (*C*) qPCR for NLRC4 and iPLA2 in infected BMDMs of the indicated genotypes showing reversal of NLRC4 and iPLA2 down-regulation in TRIF$^{-/-}$ BMDMs. BMDMs were infected with 25 MOI log phase *S.* Typhimurium for 30 min followed by gentamicin protection as in *A*. (*D*) qPCR for NLRC4 and iPLA2 in BMDMs infected with log phase *S.* Typhimurium or treated with purified recombinant IFN-β (1,000 U/mL) for the indicated times. (*E*) qPCR showing that NLRC4 and iPLA2 gene expression is not down-regulated below baseline (dashed line set to 1) in IFNAR$^{-/-}$ BMDMs. WT and IFNAR$^{-/-}$ BMDMs were infected with log phase *S.* Typhimurium as in *A*. (*F*) qPCR for NLRC4 and iPLA2 in BMDMs exposed to supernatant (i.e., secreted factors) derived from *Salmonella*-infected BMDMs. WT and IFNAR$^{-/-}$ BMDMs were treated with filter-sterilized supernatants from log phase *S.* Typhimurium-infected WT BMDMs for the indicated times and expression of NLRC4 and iPLA2 was analyzed. (*G*) Estimation of intracellular LPC in BMDMs of the indicated genotypes infected with 20 MOI log phase *S.* Typhimurium for 30 min followed by gentamicin protection for the indicated times. LPC concentration in cell lysates was measured by ELISA. The level of LPC in uninfected BMDMs of each genotype was set at 100% (dashed line) and LPC levels postinfection are represented as % change in LPC compared to uninfected BMDMs of the respective genotype. Hpi, hours postinfection. Data are representative of three (*A*–*F*) or two independent experiments (*G*). Error bars are mean ± SD of triplicates. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. ns, not significant.

is possible that through one or more of these or yet unidentified mechanisms, factors induced by IFN-β down-regulate expression of NLRC4 and iPLA2.

Our findings indicate that type 1 IFN represses expression of NLRC4 and iPLA2. Therefore, we assessed the effect of IFNAR signaling on caspase-1 activation and LPC which lie directly downstream of NLRC4 and iPLA2, respectively. Consistent with reduced NLRC4 expression, IFN-β-treated cells showed reduced cell death as measured by LDH release (*SI Appendix*, Fig. S4*A*) and reduced caspase-1 activation as evidenced by reduced amounts of cleaved caspase-1 (p20) relative to that of procaspase-1 (p46) in response to *S.* Typhimurium infection (*SI Appendix*, Fig. S4*B*). Measurement of intracellular LPC showed that consistent with the kinetics of flagellin down-regulation, intracellular LPC levels decline over time in *S.* Typhimurium-infected WT BMDMs with a significant decrease by 48 h (Fig. 5*G*). In contrast, IFNAR$^{-/-}$ BMDMs do not show a decline in intracellular LPC over time (Fig. 5*G*). Interestingly and unexpectedly, Caspase-1/11$^{-/-}$ BMDMs showed a faster decline in intracellular LPC levels compared to WT BMDMs with a significant decrease in LPC in Caspase-1/11$^{-/-}$ cells by 24 h postinfection and a further reduction at 48 h, suggesting that caspase-1 activity is upstream of LPC and maintains intracellular LPC expression. Together these data suggest that IFNAR signaling leads to a decrease in intracellular levels of LPC, likely due to repression of either a NLRC4/Caspase-1/LPC axis and/or direct repression of iPLA2 expression downstream of IFNAR (*SI Appendix*, Fig. S9*C*).

NLRC4 and iPLA2 Regulate Expression of Flagellin by Intracellular *Salmonella*. We next examined if inhibition of a NLRC4/Caspase-1 and/or iPLA2/LPC axis downstream of IFNAR contributes to down-regulation of flagellin by intracellular *Salmonella*. The reversal of flagellin down-regulation in IFNAR$^{-/-}$ BMDMs was abrogated and flagellin was down-regulated to an extent similar to that seen in WT BMDMs if either caspase-1 or iPLA2 activity was inhibited in IFNAR$^{-/-}$ BMDMs (Fig. 6 *A* and *B*). These results indicate that the effect of IFNAR signaling on *Salmonella* flagellin expression is mediated through repression of caspase-1 and iPLA2 activity and these factors are the executioners of flagellin down-regulation downstream of IFNAR. Consistent with a role for NLRC4 and caspase-1, intracellular *Salmonella* recovered from NLRC4$^{-/-}$ and Caspase-1/11$^{-/-}$ BMDMs showed nearly complete abrogation of flagellin expression both at the mRNA and the protein level at 24 h postinfection compared to those obtained from WT and ASC$^{-/-}$ BMDMs (Fig. 6*C*). NLRP3 activation is dependent on the

adapter protein ASC; therefore, these results suggest that NLRP3 does not play a role in control of flagellin expression, and caspase-1 activation by NLRC4 independent of ASC maintains flagellin expression by intracellular *Salmonella*. A similar dependence on NLRC4 and caspase-1 was observed in situ using a *S*. Typhimurium 14028 *fliC*-GFP reporter strain (*SI Appendix*, Fig. S5 *A–D*). In the absence of caspase-1 and caspase-11, *Salmonella* showed a faster time-dependent down-regulation of intracellular flagellin expression both in primary BMDMs and immortalized BMDMs (iBMDMs) (Fig. 6*C* and *SI Appendix*, Fig. S6*A*). Whereas the kinetics of flagellin down-regulation by *Salmonella* in WT iBMDMs was delayed (*SI Appendix*, Fig. S6*A*; note that flagellin in WT iBMDMs is not down-regulated even at 48 h) compared to primary BMDMs (Fig. 1*A*), clear down-regulation at 24 and 48 h was observed in Caspase-1/11$^{-/-}$ iBMDMs compared to WT iBMDMs (*SI Appendix*, Fig. S6*A*). Consistent with a requirement for caspase-1 in sustaining flagellin expression by intracellular *Salmonella*, SipB-deficient or PrgJ-deficient bacteria, which are unable to activate caspase-1 efficiently (*SI Appendix*, Fig. S1*A*) (32, 33), showed a decrease in flagellin expression at 24 h and 48 h compared to their respective parent WT *S*. Typhimurium strain (*SI Appendix*, Fig. S6*B*). These NLRC4/Caspase-1-dependent effects on flagellin down-regulation are independent of intracellular phagosomal pH because there are no differences in acidification of phagosomes between WT and Caspase-1/11$^{-/-}$ macrophages during Gram-negative bacterial infection (49). These effects were also independent of macrophage pyroptosis because intracellular *Salmonella* recovered from Gasdermin D$^{-/-}$ BMDMs which activate caspase-1 normally but are protected from pyroptotic membrane rupture (41, 42), showed flagellin down-regulation kinetics identical to bacteria residing in WT BMDMs (*SI Appendix*, Fig. S6*C*). Further, growth phase of infecting *Salmonella* did not affect the kinetics of flagellin down-regulation in BMDMs, even though stationary phase bacteria, as expected, induced lower cell death (*SI Appendix*, Fig. S7 *A–D*). Finally, flagellin down-regulation was not dependent on NLRP3 or Caspase-11 with identical kinetics of down-regulation being observed in WT, NLRP3$^{-/-}$, and Caspase-11$^{-/-}$ BMDMs (*SI Appendix*, Fig. S7 *D* and *E*). Together these results indicate that a NLRC4/Caspase-1 axis sustains expression of flagellin by intracellular *Salmonella*.

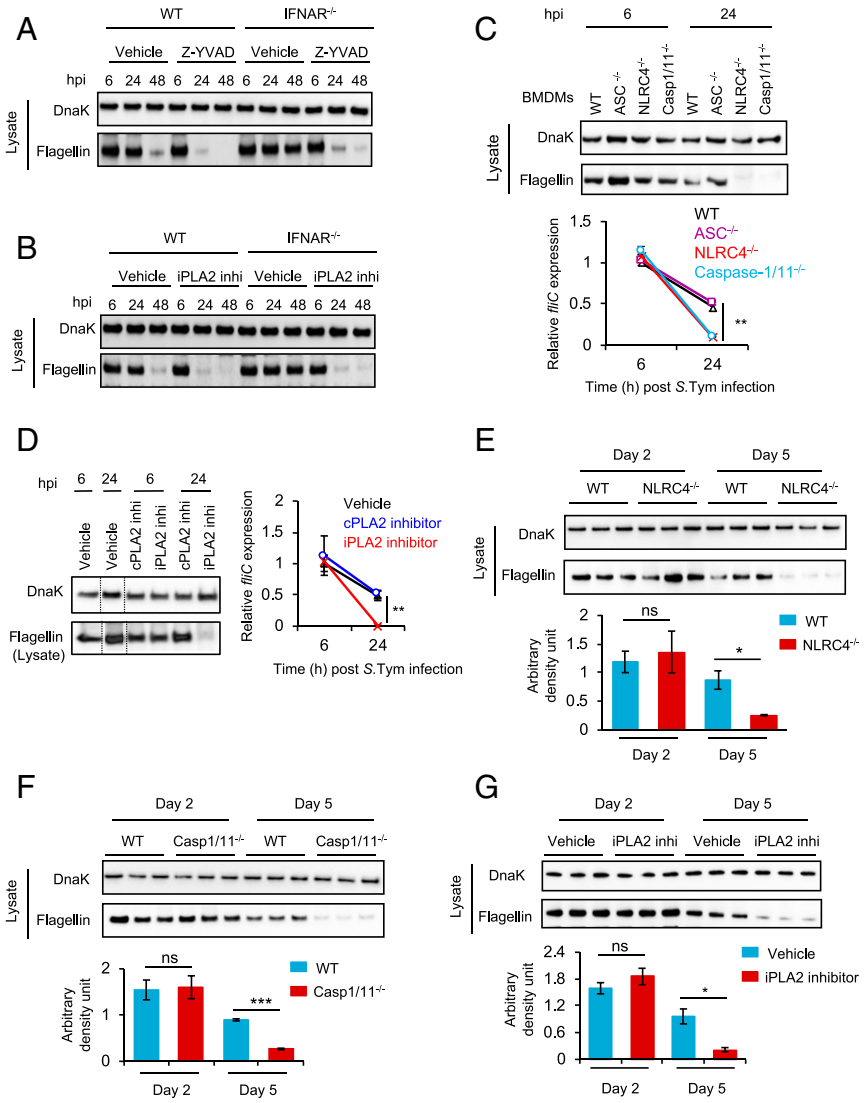


Fig. 6. NLRC4 inflammasome and iPLA2 activity controls expression of flagellin by intracellular *Salmonella*. (*A* and *B*) Immunoblot for flagellin and DnaK expressed by intracellular *Salmonella* obtained from BMDMs treated with caspase-1-specific (*A*) or iPLA2-specific (*B*) inhibitors. WT or IFNAR$^{-/-}$ BMDMs were treated with either vehicle (DMSO), the caspase-1-specific inhibitor zYVADfmk (100 μM) (*A*), or the iPLA2-specific inhibitor FKGK-11 (50 μM) (*B*) for 30 min prior to infection with 25 MOI log phase *S*. Typhimurium for 30 min. Intracellular *Salmonella* were harvested at 6, 24, and 48 h after infection. (*C*) Immunoblot (*Top*) and qPCR (*Bottom*) for flagellin expressed by intracellular *Salmonella* obtained from BMDMs of the indicated genotypes. BMDMs were infected with 25 MOI log phase *S*. Typhimurium for 30 min followed by gentamicin protection. Intracellular *Salmonella* were recovered at 6 and 24 h postinfection. (*D*) Immunoblot (*Left*) and qPCR (*Right*) for flagellin expressed by intracellular *Salmonella* obtained from BMDMs treated with PLA2 inhibitors. WT BMDMs were treated with either vehicle (DMSO) or inhibitors selective for cPLA2 (pyrrophenone, 1 μM) and iPLA2 (FKGK-11, 50 μM) for 30 min prior to infection with 25 MOI log phase *S*. Typhimurium for 30 min. Intracellular *Salmonella* were harvested at 6 and 24 h after infection. *fliC* gene expression was normalized to *dnaK*. (*E* and *F*) Immunoblot (*Top*) and corresponding densitometric quantification (*Bottom*) for flagellin expressed by intracellular *Salmonella* obtained from spleens of mice of the indicated genotypes. WT and NLRC4$^{-/-}$ mice (*E*) or WT and caspase-1/11$^{-/-}$ mice (*F*) were infected i.p. with 200 CFU log phase *S*. Typhimurium. On days 2 and 5 postinfection, flagellin and DnaK expression by intracellular *Salmonella* harvested from adherence-purified monocytes was analyzed by immunoblotting. Band intensities of flagellin were normalized to DnaK using ImageJ. (*G*) Immunoblot (*Top*) and corresponding densitometric quantification (*Bottom*) for flagellin expressed by intracellular *Salmonella* obtained from spleens of mice treated with iPLA2-specific inhibitor (FKGK-18; 20 mg/kg body weight) or vehicle (DMSO) and infected i.p. with 200 CFU log phase *S*. Typhimurium. On days 2 and 5 postinfection, intracellular *Salmonella* were recovered from adherent splenocytes and flagellin and DnaK expression were analyzed. Band intensities of flagellin were normalized to DnaK using ImageJ. Data are representative of two (*A* and *B*) or three (*C–G*) independent experiments. Error bars are mean ± SD of triplicates. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. ns, not significant.

We then further examined the relationship between lysophospholipids and expression of flagellin by intracellular *Salmonella*. Primary BMDMs were infected with *Salmonella* in the presence or absence of inhibitors selective for cPLA2 or iPLA2 to inhibit the biogenesis of lysophospholipids generated by these enzymes. Treatment with the iPLA2-selective inhibitor FKGK-11, which specifically inhibits iPLA2 without affecting caspase-1 activation (50) or macrophage cell death (Fig. 3 *D*, *Right*), abrogated expression of flagellin by intracellular bacteria both at the mRNA and the protein levels at 24 h (Fig. 6*D*). Treatment with the cPLA2-selective inhibitor pyrrophenone (51) had no effect (Fig. 6*D*). Similar results were obtained in iBMDMs (*SI Appendix*, Fig. S6*D*), suggesting that iPLA2-generated lysophospholipids support expression of flagellin by intracellular *Salmonella*. The intracellular levels of LPC also correlated well with flagellin expression by *Salmonella* isolated from WT macrophages and macrophages lacking different inflammasome components. While the levels of LPC decreased only at 48 h postinfection in WT, NLRP3$^{-/-}$, and Caspase-11$^{-/-}$ macrophages, NLRC4$^{-/-}$ and Caspase-1/11$^{-/-}$ macrophages showed a faster decline in intracellular LPC with a significant reduction by 24 h postinfection (*SI Appendix*, Fig. S8 *A* and *B*). Consistent with the faster reduction in LPC, intracellular *Salmonella* down-regulated flagellin faster in NLRC4$^{-/-}$ and Caspase-1/11$^{-/-}$ macrophages as compared to WT, NLRP3$^{-/-}$, and Caspase-11$^{-/-}$ macrophages (*SI Appendix*, Fig. S7 *D* and *E*).

Salmonella populations can be stochastic and heterogeneous in nature (52), so we next asked if flagellin down-regulation by intracellular *Salmonella* in WT macrophages over time (Fig. 1*A*) could simply be due to a preferential selection of cells infected with flagellin-low or perhaps PrgJ-low *Salmonella* in the invading population of bacteria as these cells are likely to not die by pyroptosis (see schematic in *SI Appendix*, Fig. S6*E*), rather than an adaptation by *Salmonella* to an infection-induced decrease in host NLRC4 and iPLA2. Our collective data argued against this possibility. Firstly, inhibition of iPLA2 in WT BMDMs did not affect *Salmonella*-induced cell death (Fig. 3 *D*, *Right*), but still led to faster down-regulation of flagellin expression by intracellular *Salmonella* (Fig. 6*D*), suggesting that flagellin expression is dependent on iPLA2-generated lipids and not on pyroptosis. Secondly, as shown in *SI Appendix*, Fig. S6*C*, intracellular bacteria from Gasdermin D$^{-/-}$ BMDMs which are protected from pyroptotic membrane rupture show flagellin down-regulation kinetics identical to bacteria from WT BMDMs. Thirdly, stationary phase bacteria induced lower cell death but showed flagellin down-regulation kinetics identical to that of log phase bacteria (*SI Appendix*, Fig. S7 *A–D*). Fourthly, PrgJ-deficient *Salmonella* down-regulated flagellin intracellularly with kinetics identical to SipB-deficient bacteria when compared to their respective parental congenic WT counterparts, strongly suggesting that lack of caspase-1 activation due to PrgJ deficiency leads to flagellin down-regulation and is independent of PrgJ expression per se (*SI Appendix*, Fig. S6*B*). Finally, if selection bias was at play, then we would expect NLRC4$^{-/-}$ and Caspase-1/11$^{-/-}$ macrophages to retain more flagellin-high bacteria intracellularly over time because of the resistance of these genotypes to pyroptosis and consequently show a slower down-regulation of flagellin compared to WT macrophages (see schematic in *SI Appendix*, Fig. S6*E*), an outcome opposite to the observed outcome (Fig. 6*C* and *SI Appendix*, Fig. S5). Of note, although NLRC4 and iPLA2 expression in WT BMDMs was down-regulated early on (i.e., maximally by 10 h postinfection at the mRNA level; Fig. 4 *A* and *B*), flagellin down-regulation peaked at 48 h postinfection (Fig. 1*A*). The abundance of cellular lysophospholipids and the time it takes to deplete the preexisting intracellular lipid pool in macrophages (Fig. 5*G* and *SI Appendix*, Fig. S8 *A* and *B*) may contribute to the delayed kinetics of flagellin down-regulation.

Finally, we examined the role of the NLRC4/Caspase-1 and iPLA2 axes in regulating flagellin expression by intracellular bacteria during in vivo infection. WT, NLRC4$^{-/-}$, Caspase-1/11$^{-/-}$ mice or mice treated with the iPLA2-specific in vivo inhibitor, FKGK-18 (53), were infected i.p. with *S*. Typhimurium and killed at days 2 and 5 postinfection. Bacteria were recovered from the spleen and flagellin expression was analyzed. Intracellular bacteria obtained from NLRC4$^{-/-}$ and Caspase-1/11$^{-/-}$ spleens showed markedly greater down-regulation of flagellin compared to those recovered from WT spleens (Fig. 6 *E* and *F*). Reduced flagellin expression on day 5 in NLRC4$^{-/-}$ and Caspase-1/11$^{-/-}$ mice was associated with increased bacterial burden in the spleens of these mice (*SI Appendix*, Fig. S8*C*). Similarly, intracellular bacteria recovered from spleens of iPLA2 inhibitor-treated mice showed significantly lower expression of flagellin at day 5 compared to those treated with vehicle (Fig. 6*G*). These results indicate that NLRC4/Caspase-1 and iPLA2 activity sustains expression of flagellin by intracellular *Salmonella* both in vitro and in vivo.

Discussion

Coevolution of pathogens and the host immune system has resulted in complex evolutionary adaptations that contribute to host defense and an array of immune evasion mechanisms in the microbes. Pyroptosis, a caspase-1-mediated form of inflammatory cell death induced upon sensing of flagellin by NLRC4, is essential for clearance of pathogenic *Salmonella* from the host and is circumvented by the pathogen by down-regulating the expression of flagellin at later stages of infection. Although this set of linked events is well described, the molecular mechanisms at play in prompting the pathogen to switch to a flagellin-low phenotype in vivo and establish an intracellular survival niche within macrophages are incompletely understood. It is believed based on in vitro studies of bacteria exposed to various perturbations in growth medium, that conditions associated with the SCV such as acidic pH and low Mg^{2+} suppress expression of flagellin (20, 21); however, the contribution of these factors in controlling flagellin expression by intracellular *Salmonella* inside macrophages is controversial (20, 24–26). Here, we provide evidence that a natural type 1 IFN-dependent host negative feedback response to bacterial infection enables down-regulation of flagellin by *Salmonella* inside macrophages (*SI Appendix*, Fig. S9*C*). Interestingly, during early infection of macrophages with *Salmonella*, NLRC4 inflammasome-dependent pyroptosis is necessary and sufficient for generating a host lysophospholipid stimulus that increases expression of biologically active monomeric flagellin by extracellular bacteria in a feedforward manner (*SI Appendix*, Fig. S9*B*). The increased flagellin can confer an early advantage to the pathogen by promoting TLR-5-dependent systemic dissemination of *Salmonella* (54); however, our data show that the feedforward circuit generates bacteria which are more potent at inducing pyroptosis of healthy macrophages (Fig. 2*F*), a modulation that is known to result in neutrophil-mediated killing and clearance of *Salmonella* from the host (17) (*SI Appendix*, Fig. S9*B*). This positive feedback loop is, however, later kept in check by type 1 IFN-dependent shutdown of NLRC4 and iPLA2 expression in macrophages, creating an intracellular environment low in caspase-1 activity and lysophospholipids that *Salmonella* adapts to for flagellin down-regulation inside macrophages (*SI Appendix*, Fig. S9*C*). Overall, our findings suggest that *Salmonella* has evolved to coopt NLRC4 activation to initially enhance production of extracellular flagellin that activates TLR-5, which along with other TLRs promotes systemic spread of the pathogen (54, 55) at the risk of NLRC4-mediated clearance, and later on benefit from the IFN-induced decrease in NLRC4 and lysophospholipid production to down-regulate flagellin intracellularly within macrophages. These data also identify a role for NLRC4 inflammasome activity, which is classically implicated in the detection and

clearance of a variety of bacterial pathogens, as a regulator of temporal and biphasic expression of its own bacterial ligand.

One consequence of the IFN-induced decrease in NLRC4 for the host is that it limits infection-induced pyroptosis (*SI Appendix*, Fig. S4). Down-regulation of NLRC4 expression may therefore be a preemptive host measure to restrain cell death and preserve a macrophage pool for subsequent priming of an adaptive immune response (56). It may also, along with direct down-regulation of iPLA2, be a host strategy to limit generation of LPC and prevent overt inflammation. This is because LPC released by dying cells acts as a chemoattractant for macrophages, which is believed to be beneficial for effective removal of cell corpses (57); however, unchecked inflammatory cell recruitment can exacerbate inflammation and cause tissue-damaging effects. LPC can also amplify TLR-activated inflammatory responses (58), an excess of which can be detrimental to the host. A recent study demonstrated reduced NLRC4 expression in LPS-treated human monocytes (59) and in a three-dimensional organotypic model of human intestinal mucosa stimulated with *S*. Typhi (60), suggesting that the IFN-dependent NLRC4^{lo} state described here might also be relevant in human cells. Although our data in macrophages show that IFNAR signaling potently down-regulates NLRC4 and iPLA2 to subbaseline levels, it is important to note that in vivo, in addition to type 1 IFN, other interferons derived from various cellular sources could also possibly contribute to establishment of a NLRC4-, iPLA2-, and LPC-low environment. Future studies are required to identify the molecular details of type 1 IFN-induced repression of NLRC4 and iPLA2 and possible involvement of other interferons in this process.

For the pathogen, an important outcome of a NLRC4- and iPLA2-low intracellular environment depleted of lysophospholipids is its switching to a flagellin-low phenotype. Given that flagellin is a potent mediator of innate and inflammatory responses (5), a target of antibodies and T cells during infection (61, 62), and can even affect the suppressive function of T regulatory cells (63), down-regulation of flagellin could allow the pathogen to escape immune control. Consistent with this notion, it had been shown that *Salmonella* designed to persistently express flagellin is cleared in a NLRC4/Caspase-1-dependent manner and is hence attenuated in vivo (17). The data provided here show that flagellin down-regulation by intracellular *Salmonella* is accelerated in NLRC4$^{-/-}$ as well as Caspase-1/11$^{-/-}$ mice (Fig. 6 *E* and *F*) and is reversed in IFNAR$^{-/-}$ mice (Fig. 1*E*). It is notable that flagellin expression by intracellular *Salmonella* during the systemic phase of infection in these mice correlates inversely with the susceptibility of these mice to *Salmonella* infection. NLRC4$^{-/-}$ as well as Caspase-1/11$^{-/-}$ mice are more susceptible and are reported to display higher bacterial burdens than WT mice (64, 65) while IFNAR$^{-/-}$ mice are less susceptible and display lower bacterial burdens than WT mice (29). In addition, reduced serum levels of lysophospholipids have been reported in patients with gastroenteritis caused by *Salmonella* (66), implying that establishment of successful infection by *Salmonella* might be strongly linked to its ability to down-regulate flagellin expression under conditions of lowered iPLA2 expression.

Given that macrophages undergo rapid caspase-1-dependent pyroptosis in response to *Salmonella* infection, an interesting issue relates to the nature of cells that survive the initial onslaught of infection by extracellular bacteria and subsequently provide a niche for intracellular residence and flagellin down-regulation by *Salmonella*. Although caspase-1 activation is believed to be an all-or-none digital response at the single cell level using current methods (67), all prevailing methods for measuring caspase-1 activity are insensitive to weak or local activations of caspase-1. Therefore, it is possible that infected cells with a weak or subthreshold level of caspase-1 activation that is insufficient to commit macrophages to pyroptosis survive the initial onslaught of infection. Moreover, our data suggest that type 1 IFN produced by macrophages early during infection likely fosters a NLRC4^{lo}i-PLA2^{lo} state in the surviving cells which promotes flagellin down-regulation by the pathogen (Figs. 1*B* and 5*F*). Given that levels of intracellular flagellin correlate inversely with systemic bacterial loads, it is possible that these NLRC4- and iPLA2-low cells might serve as an ideal reservoir for stealthily replicating *Salmonella* and ultimately potentiate dissemination of the pathogen to secondary lymphoid organs. Recent single-cell RNA-Seq studies suggest that variable gene expression in infected host cells shapes different functional cellular states, some of which favor the establishment of a long-term intracellular niche, and others that allow *Salmonella* to escape intracellular immune activity and proliferate (68). Although current single-cell RNA-Seq methods are restricted to profiling eukaryotic transcripts, future technical improvements of the dual RNA-Seq technique which profiles gene expression in host and bacteria simultaneously (69) may permit the correlation of flagellin and other bacterial virulence factors with host cell heterogeneity and reprogramming.

Another interesting aspect of the intracellular flagellin regulatory circuit we describe here is that caspase-1 is upstream of LPC and can control the levels of intracellular LPC (Fig. 5*G*). Because caspase-1 activation is traditionally believed to induce pyroptotic cell death of macrophages, an outstanding question concerns the mechanism by which caspase-1 regulates the levels of intracellular LPC independent of pyroptosis. In this regard, it is noteworthy that a branch of lipid synthesis during innate immune activation relies on caspase-1 whereby caspase-1 induces the activation of sterol regulatory element binding proteins (SREBPs) which in turn promote expression of genes involved in lipid synthesis (70). SREBP-1 activation leads to production of phospholipids (71) which are substrates for production of lysophospholipids. Alternatively, SREBP-2 can regulate the expression of iPLA2. The 5′ flanking region of the iPLA2 gene is reported to contain a sterol regulatory element (SRE) that can bind SREBP-2, and mutant cells that constitutively generate mature SREBP proteins exhibit increased iPLA2 expression and activity (72). Through these or a yet unidentified mechanism(s), caspase-1 activity could control intracellular lysophospholipid levels.

Taken together, our data identify a type 1 IFN-dependent host mechanism that fosters a NLRC4- and iPLA2-low cellular state which is coopted by *Salmonella* to switch to a flagellin-low phenotype inside macrophages. Our findings also identify a mode of innate immune regulation whereby an innate sensing mechanism, that is, NLRC4 inflammasome activity, regulates temporal expression of its own microbial ligand, and add to our understanding of the complex evolutionary adaptations that shape host–pathogen cross-talk.

Materials and Methods

Detailed information on experimental models (bacterial strains, cell lines, mice), reagents, assays, and statistical analysis is presented in *SI Appendix*, *SI Materials and Methods*. *S*. Typhimurium SL1344 grown to log phase in LB were used in all experiments unless mentioned otherwise. All animal experiments were approved by the Institute for Systems Biology's Institutional Animal Care and Use Committee.

Data Availability. All study data are included in the article and *SI Appendix*.

ACKNOWLEDGMENTS. This work was supported by the Institute for Systems Biology, and in part by the National Institute of Immunology (funded by the Department of Biotechnology, Government of India) and funds from the Steven and Alexandra Cohen Foundation to N.S. We thank Prof. Kazuhiro Kutsukake for *S*. Typhimurium strain KK1004 and its *flhD*::Tn10 derivative; Prof. Emmanuelle Charpentier for *S*. Typhimurium SL1344 and its SipB-negative derivative; Dr. Brad Cookson for *S*. Typhimurium 14028 expressing *fliC*-GFP; Dr. Eicke Latz for WT and Caspase-1/11-deficient bone marrow-derived macrophage cell lines; Dr. Russell Vance for bones from Gasdermin D$^{-/-}$ mice; Dr. Vishva Dixit for NLRC4$^{-/-}$ and Caspase 1/11$^{-/-}$ mice; and Drs. Teresa Thurston and David Holden for critical reading of the manuscript.

1. S. W. Brubaker, K. S. Bonham, I. Zanoni, J. C. Kagan, Innate immune pattern recognition: A cell biological perspective. *Annu. Rev. Immunol.* **33**, 257–290 (2015).
2. N. Arpaia *et al*., TLR signaling is required for Salmonella typhimurium virulence. *Cell* **144**, 675–688 (2011).
3. K. D. Smith *et al*., Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial motility. *Nat. Immunol.* **4**, 1247–1253 (2003).
4. E. A. Miao *et al*., Cytoplasmic flagellin activates caspase-1 and secretion of interleukin 1beta via Ipaf. *Nat. Immunol.* **7**, 569–575 (2006).
5. H. Zeng *et al*., Flagellin is the major proinflammatory determinant of enteropathogenic Salmonella. *J. Immunol.* **171**, 3668–3674 (2003).
6. E. M. Kofoed, R. E. Vance, Innate immune recognition of bacterial ligands by NAIPs determines inflammasome specificity. *Nature* **477**, 592–595 (2011).
7. Y. Zhao *et al*., The NLRC4 inflammasome receptors for bacterial flagellin and type III secretion apparatus. *Nature* **477**, 596–600 (2011).
8. M. Rayamajhi, D. E. Zak, J. Chavarria-Smith, R. E. Vance, E. A. Miao, Cutting edge: Mouse NAIP1 detects the type III secretion system needle protein. *J. Immunol.* **191**, 3986–3989 (2013).
9. J. Yang, Y. Zhao, J. Shi, F. Shao, Human NAIP and mouse NAIP1 recognize bacterial type III secretion needle protein for inflammasome activation. *Proc. Natl. Acad. Sci. U.S.A.* **110**, 14408–14413 (2013).
10. V. M. Reyes Ruiz *et al*., Broad detection of bacterial type III secretion system and flagellin proteins by the human NAIP/NLRC4 inflammasome. *Proc. Natl. Acad. Sci. U.S.A.* **114**, 13242–13247 (2017).
11. Z. Hu *et al*., Structural and biochemical basis for induced self-propagation of NLRC4. *Science* **350**, 399–404 (2015).
12. L. Zhang *et al*., Cryo-EM structure of the activated NAIP2-NLRC4 inflammasome reveals nucleated polymerization. *Science* **350**, 404–409 (2015).
13. P. Broz *et al*., Redundant roles for inflammasome receptors NLRP3 and NLRC4 in host defense against Salmonella. *J. Exp. Med.* **207**, 1745–1755 (2010).
14. M. A. Wynosky-Dolfi *et al*., Oxidative metabolism enables Salmonella evasion of the NLRP3 inflammasome. *J. Exp. Med.* **211**, 653–668 (2014).
15. L. Franchi *et al*., Cytosolic flagellin requires Ipaf for activation of caspase-1 and interleukin 1beta in salmonella-infected macrophages. *Nat. Immunol.* **7**, 576–582 (2006).
16. L. A. Knodler *et al*., Noncanonical inflammasome activation of caspase-4/caspase-11 mediates epithelial defenses against enteric bacterial pathogens. *Cell Host Microbe* **16**, 249–256 (2014).
17. E. A. Miao *et al*., Caspase-1-induced pyroptosis is an innate immune effector mechanism against intracellular bacteria. *Nat. Immunol.* **11**, 1136–1142 (2010).
18. L. A. Cummings, S. L. Barrett, W. D. Wilkerson, I. Fellnerova, B. T. Cookson, FliC-specific CD4+ T cell responses are restricted by bacterial regulation of antigen expression. *J. Immunol.* **174**, 7929–7938 (2005).
19. J. J. Bijlsma, E. A. Groisman, The PhoP/PhoQ system controls the intramacrophage type three secretion system of Salmonella enterica. *Mol. Microbiol.* **57**, 85–96 (2005).
20. P. Adams *et al*., Proteomic detection of PhoPQ- and acid-mediated repression of Salmonella motility. *Proteomics* **1**, 597–607 (2001).
21. L. A. Cummings, W. D. Wilkerson, T. Bergsbaken, B. T. Cookson, In vivo, fliC expression by Salmonella enterica serovar Typhimurium is heterogeneous, regulated by ClpX, and anatomically restricted. *Mol. Microbiol.* **61**, 795–809 (2006).
22. E. García Véscovi, F. C. Soncini, E. A. Groisman, Mg2+ as an extracellular signal: Environmental regulation of Salmonella virulence. *Cell* **84**, 165–174 (1996).
23. F. C. Soncini, E. García Véscovi, F. Solomon, E. A. Groisman, Molecular basis of the magnesium deprivation response in Salmonella typhimurium: Identification of PhoP-regulated genes. *J. Bacteriol.* **178**, 5092–5099 (1996).
24. D. Drecktrah, L. A. Knodler, R. Ireland, O. Steele-Mortimer, The mechanism of Salmonella entry determines the vacuolar environment and intracellular gene expression. *Traffic* **7**, 39–51 (2006).
25. M. Rathman, M. D. Sjaastad, S. Falkow, Acidification of phagosomes containing Salmonella typhimurium in murine macrophages. *Infect. Immun.* **64**, 2765–2773 (1996).
26. N. Martin-Orozco *et al*., Visualization of vacuolar acidification-induced transcription of genes of pathogens inside macrophages. *Mol. Biol. Cell* **17**, 498–510 (2006).
27. G. Sano *et al*., Flagella facilitate escape of Salmonella from oncotic macrophages. *J. Bacteriol.* **189**, 8224–8232 (2007).
28. G. Guarda *et al*., Type I interferon inhibits interleukin-1 production and inflammasome activation. *Immunity* **34**, 213–223 (2011).
29. N. Robinson *et al*., Type I interferon induces necroptosis in macrophages during infection with Salmonella enterica serovar Typhimurium. *Nat. Immunol.* **13**, 954–962 (2012).
30. J. E. Karlinsey, J. Lonner, K. L. Brown, K. T. Hughes, Translation/secretion coupling by type III secretion systems. *Cell* **102**, 487–497 (2000).
31. J. L. Tenthorey *et al*., NLRC4 inflammasome activation is NLRP3- and phosphorylation-independent during infection and does not protect from melanoma. *J. Exp. Med.* **217**, e20191736 (2020).
32. D. Hersh *et al*., The Salmonella invasin SipB induces macrophage apoptosis by binding to caspase-1. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 2396–2401 (1999).
33. E. A. Miao *et al*., Innate immune detection of the type III secretion apparatus through the NLRC4 inflammasome. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 3076–3080 (2010).
34. A. Qadri, S. Ghosh, S. Upadhyay, G. P. Talwar, Monoclonal antibodies against flagellar antigen of Salmonella typhi. *Hybridoma* **8**, 353–360 (1989).
35. N. Subramanian, A. Qadri, Lysophospholipid sensing triggers secretion of flagellin from pathogenic salmonella. *Nat. Immunol.* **7**, 583–589 (2006).
36. J. A. Hagar, D. A. Powell, Y. Aachoui, R. K. Ernst, E. A. Miao, Cytoplasmic LPS activates caspase-11: Implications in TLR4-independent endotoxic shock. *Science* **341**, 1250–1253 (2013).
37. N. Kayagaki *et al*., Noncanonical inflammasome activation by intracellular LPS independent of TLR4. *Science* **341**, 1246–1249 (2013).
38. S. Shivcharan, J. Yadav, A. Qadri, Host lipid sensing promotes invasion of cells with pathogenic Salmonella. *Sci. Rep.* **8**, 15501 (2018).
39. C. D. Ellermeier, J. R. Ellermeier, J. M. Slauch, HilD, HilC and RtsA constitute a feed forward loop that controls expression of the SPI1 type three secretion system regulator hilA in Salmonella enterica serovar Typhimurium. *Mol. Microbiol.* **57**, 691–705 (2005).
40. H. M. Singer, C. Kühne, J. A. Deditius, K. T. Hughes, M. Erhardt, The Salmonella Spi1 virulence regulatory protein HilD directly activates transcription of the flagellar master operon flhDC. *J. Bacteriol.* **196**, 1448–1457 (2014).
41. N. Kayagaki *et al*., Caspase-11 cleaves gasdermin D for non-canonical inflammasome signalling. *Nature* **526**, 666–671 (2015).
42. J. Shi *et al*., Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. *Nature* **526**, 660–665 (2015).
43. F. G. Bauernfeind *et al*., Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. *J. Immunol.* **183**, 787–791 (2009).
44. A. Sing *et al*., Bacterial induction of beta interferon in mice is a function of the lipopolysaccharide component. *Infect. Immun.* **68**, 1600–1607 (2000).
45. D. C. Otero, N. J. Fares-Frederickson, M. Xiao, D. P. Baker, M. David, IFN-β selectively inhibits IL-2 production through CREM-mediated chromatin remodeling. *J. Immunol.* **194**, 5120–5128 (2015).
46. D. Sarkar, E. S. Park, P. B. Fisher, Defining the mechanism by which IFN-beta dowregulates c-myc expression in human melanoma cells: Pivotal role for human polynucleotide phosphorylase (hPNPaseold-35). *Cell Death Differ.* **13**, 1541–1553 (2006).
47. Z. Ma, H. Qin, E. N. Benveniste, Transcriptional suppression of matrix metalloproteinase-9 gene expression by IFN-gamma and IFN-beta: Critical role of STAT-1alpha. *J. Immunol.* **167**, 5150–5159 (2001).
48. F. W. McNab *et al*., Type I IFN induces IL-10 production in an IL-27-independent manner and blocks responsiveness to IFN-γ for production of IL-12 and bacterial killing in Mycobacterium tuberculosis-infected macrophages. *J. Immunol.* **193**, 3600–3612 (2014).
49. A. Sokolovska *et al*., Activation of caspase-1 by the NLRP3 inflammasome regulates the NADPH oxidase NOX2 to control phagosome function. *Nat. Immunol.* **14**, 543–553 (2013).
50. C. Baskakis *et al*., Synthesis of polyfluoro ketones for selective inhibition of human phospholipase A2 enzymes. *J. Med. Chem.* **51**, 8027–8037 (2008).
51. K. Seno *et al*., Pyrrolidine inhibitors of human cytosolic phospholipase A2. Part 2: Synthesis of potent and crystallized 4-triphenylmethylthio derivative 'pyrrophenone'. *Bioorg. Med. Chem. Lett.* **11**, 587–590 (2001).
52. M. K. Stewart, L. A. Cummings, M. L. Johnson, A. B. Berezow, B. T. Cookson, Regulation of phenotypic heterogeneity permits Salmonella evasion of the host caspase-1 inflammatory response. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 20742–20747 (2011).
53. T. Ali *et al*., Characterization of FKGK18 as inhibitor of group VIA Ca2+-independent phospholipase A2 (iPLA2β): Candidate drug for preventing beta-cell apoptosis and diabetes. *PLoS One* **8**, e71748 (2013).
54. S. Uematsu *et al*., Detection of pathogenic intestinal bacteria by toll-like receptor 5 on intestinal CD11c+ lamina propria cells. *Nat. Immunol.* **7**, 868–874 (2006).
55. S. Talbot *et al*., Toll-like receptor 4 signalling through MyD88 is essential to control Salmonella enterica serovar typhimurium infection, but not for the initiation of bacterial clearance. *Immunology* **128**, 472–483 (2009).
56. M. M. McDaniel, L. C. Kottyan, H. Singh, C. Pasare, Suppression of inflammasome activation by IRF8 and IRF4 in cDCs is critical for T cell priming. *Cell Rep.* **31**, 107604 (2020).
57. K. Lauber *et al*., Apoptotic cells induce migration of phagocytes via caspase-3-mediated release of a lipid attraction signal. *Cell* **113**, 717–730 (2003).
58. N. Sharma, A. S. Akhade, S. Ismaeel, A. Qadri, Serum-borne lipids amplify TLR-activated inflammatory responses. *J. Leukoc. Biol.*, 10.1002/JLB.3AB0720-241RR (2020).
59. F. Awad *et al*., Impact of human monocyte and macrophage polarization on NLR expression and NLRP3 inflammasome activation. *PLoS One* **12**, e0175336 (2017).
60. R. Salerno-Gonçalves, J. E. Galen, M. M. Levine, A. Fasano, M. B. Sztein, Manipulation of *Salmonella* typhi gene expression impacts innate cell responses in the human intestinal mucosa. *Front. Immunol.* **9**, 2543 (2018).
61. A. Brown, C. E. Hormaeche, The antibody response to salmonellae in mice and humans studied by immunoblots and ELISA. *Microb. Pathog.* **6**, 445–454 (1989).
62. S. J. McSorley, B. T. Cookson, M. K. Jenkins, Characterization of CD4+ T cell responses during natural infection with Salmonella typhimurium. *J. Immunol.* **164**, 986–993 (2000).
63. N. K. Crellin *et al*., Human CD4+ T cells express TLR5 and its ligand flagellin enhances the suppressive capacity and expression of FOXP3 in CD4+CD25+ T regulatory cells. *J. Immunol.* **175**, 8051–8059 (2005).
64. F. A. Carvalho *et al*., Cytosolic flagellin receptor NLRC4 protects mice against mucosal and systemic challenges. *Mucosal Immunol.* **5**, 288–298 (2012).
65. M. Lara-Tejero *et al*., Role of the caspase-1 inflammasome in Salmonella typhimurium pathogenesis. *J. Exp. Med.* **203**, 1407–1412 (2006).
66. V. K. Makarov, A. E. Leventsova, [The lipid composition of bloodserum in patients with salmonella infection and suffering of alcohol abuse]. *Klin. Lab. Diagn.*, 14–17 (2012).
67. T. Liu *et al*., Single-cell imaging of caspase-1 dynamics reveals an all-or-none inflammasome signaling response. *Cell Rep.* **8**, 974–982 (2014).
68. A. E. Saliba *et al*., Single-cell RNA-seq ties macrophage polarization to growth rate of intracellular Salmonella. *Nat. Microbiol.* **2**, 16206 (2016).
69. A. J. Westermann *et al*., Dual RNA-seq unveils noncoding RNA functions in host-pathogen interactions. *Nature* **529**, 496–501 (2016).
70. L. Gurcel, L. Abrami, S. Girardin, J. Tschopp, F. G. van der Goot, Caspase-1 activation of lipid metabolic pathways in response to bacterial pore-forming toxins promotes cell survival. *Cell* **126**, 1135–1145 (2006).
71. J. D. Horton, J. L. Goldstein, M. S. Brown, SREBPs: Activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J. Clin. Invest.* **109**, 1125–1131 (2002).
72. S. J. Seashols, A. del Castillo Olivares, G. Gil, S. E. Barbour, Regulation of group VIA phospholipase A2 expression by sterol availability. *Biochim. Biophys. Acta* **1684**, 29–37 (2004).

Supplementary Information for

Type 1 interferon-dependent repression of NLRC4 and iPLA2 licenses downregulation of *Salmonella* flagellin inside macrophages

Ajay S. Akhade[1], Shaikh M. Atif[2], Bhavana S. Lakshmi[1], Neha Dikshit[2], Kelly T. Hughes[3], Ayub Qadri[2,5], Naeha Subramanian[1,4,5,6]

Naeha Subramanian
Email: nsubrama@systemsbiology.org

Ayub Qadri
Email: ayub@nii.ac.in

This PDF file includes:

SI Materials and Methods
Figures S1 to S9
SI References

SI Materials and Methods

Contact for Reagent and Resource Sharing

All data is available in the main text or the supplementary materials. This study did not generate any unique datasets or code. Reagents described in this study are available upon request to N.S. (nsubrama@systemsbiology.org).

Experimental Model Details

Bacterial strains

Details are provided in Table S1. Bacteria were grown overnight with shaking in Luria-Bertani (LB) medium. Experiments were done using the WT *S*.Typhimurium SL1344 strain unless mentioned otherwise. In all experiments involving use of bacterial mutants, relevant congenic parental WT strains were used as a control.

Cell lines and mice

WT C57BL6 mice, TRIF$^{-/-}$ and Caspase-11$^{-/-}$ mice were obtained from Jackson laboratories. NLRC4$^{-/-}$ and Caspase-1/11$^{-/-}$ mice on the C57BL6 background were provided by Dr. Vishva Dixit (Genentech). MyD88$^{-/-}$ mice and IFNAR$^{-/-}$ mice on the C57BL6 background were obtained from Dr. Michael Gale Jr (University of Washington, Seattle). Bones from Gasdermin D$^{-/-}$ mice were provided by Dr. Russell Vance (University of California, Berkeley). All animal experiments were approved by the Institute for Systems Biology's Institutional Animal Care and Use Committee. WT and Caspase-1-deficient immortalized BMDM lines (C57BL/6 background) were provided by Dr. Eicke Latz, Institute of Innate Immunity, Bonn. Cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated FCS in a humidified atmosphere of 5% CO_2 at 37°C.

Antibodies and other reagents

Pancaspase inhibitor (zVADfmk, Cat# 219007) and caspase-1 specific inhibitor (zYVADfmk Cat# 218746) were obtained from Calbiochem. iPLA2 inhibitor (FKGK-11, Cat# 13179) and cPLA2 inhibitor (Pyrrophenone, Cat# 13294) were from Cayman Chemicals. iPLA2 inhibitor for *in vivo* use (FKGK-18, Cat# AOB4846) was acquired from Aobious. *S*.Typhimurium LPS was from Sigma (Cat# L6143). Lysophospholipids (18:1 LPA Cat# 857130, 18:1 LPC Cat# 855773) were obtained from Avanti Polar Lipids. Monoclonal antibodies to *S*.Typhi flagellin and *S*.Typhi LPS were generated in the Qadri laboratory and have been described previously (1, 2). The

following commercially available antibodies were used for immunoblotting: Rabbit antiserum to *S*.Typhimurium flagellin (Cat# 228421) and *S*.Typhimurium LPS (Cat# 227791 BD Laboratories), anti-DnaK antibody (Enzo Life Sciences, Cat# ADI-SPA-880), anti–NLRC4 (Calbiochem, Cat# 06-1125), anti–iPLA2 (Cayman, Cat# 160507), anti–NLRP3 (Adipogen, Cat# AG-20B-0014) and anti–ASC (Cat# 271054), anti–caspase–1(Cat# 56036) and anti–ERK2 (Cat# 1647, Santacruz).

Method Details

Generation of mouse macrophages

Splenic, bone marrow–derived and peritoneal macrophages from C57BL/6 mice were isolated by standard protocols (3). Peritoneal macrophages were elicited by injection of 4% thioglycollate solution and were collected by peritoneal lavage 3 days later. Adherent macrophages were prepared by culturing cells at a density of $3x10^6$ cells/well in a 6-well plate in serum-free RPMI for 2 h and removing non-adherent cells.

For isolating splenic macrophages, a single cell suspension of splenocytes was prepared, RBCs were lysed using Ack lysing buffer and cells were left for adherence in a tissue culture dish for 1 h. Non-adherent cells were removed by washing and adherent cells were scraped off and seeded in a 6 well plate at a density of $2x10^6$ cells/well.

For BMDMs, mice were euthanized, bones (femur and tibia) were harvested and flushed with ice-cold cell culture medium. RBCs were lysed using Ack lysing buffer and cells were plated in a 150 mm tissue culture dish in medium containing 50 ng/ml m-CSF. On day 3, cells were fed with an equal volume of fresh medium containing 50 ng/ml m-CSF. BMDMs were harvested on day 5 by scraping in ice-cold PBS. Cells were seeded in cell culture medium with 50 ng/ml m-CSF at a density of $2x10^6$ cells per well in a 6 well plate.

S.Typhimurium infection of macrophages and analysis of extracellular flagellin release

For all experiments, *S*.Typhimurium were grown to mid to late log phase (8-10 h) in LB broth at 37°C with shaking at 220rpm unless mentioned otherwise. Bacteria were washed with and resuspended in serum-free RPMI-1640 medium. Bacteria were incubated with macrophages at a pathogen to cell ratio of 50:1 or as indicated in the figure legends in 1 ml serum–free RPMI-1640 for 1 h at 37°C with 5% CO_2. A high MOI (50) was chosen so as to liberate sufficient amounts of the cell death-associated intracellular host stimulus responsible for enhancing flagellin expression by *Salmonella*. In some experiments, peritoneal macrophages were treated with 100 μM zVADfmk or 100 μM zYVADfmk for 30 min prior to infection. After inhibitor

treatment, macrophages were infected in presence of inhibitor for 1 h. Macrophages were also infected with *S*.Typhimurium strains lacking *SipB* or carrying transposon Tn10 insertion mutations in *fliD* (*fliD*::Tn10), *flhD* (*flhD*::Tn10) and *prgJ* (*prgJ*::Tn10) genes. When mutant strains were included in the assay, infections were synchronized by centrifuging plates at 1500 rpm for 10 min. Following incubation, bacteria were pelleted at 13,000 rpm for 5 min and supernatants were filtered through a 0.22 μm membrane to remove bacteria and/or any cellular debris. Filtered supernatants were analyzed for flagellin by immunoblotting with *S*.Typhimurium flagellin-specific polyclonal antibody (BD Laboratories). Reactive bands were visualized with Enhanced Chemiluminescence Reagent (Pierce).

Analysis of flagellin release by *S*.Typhi

Filter-sterilized supernatants from *S*.Typhimurium-infected or flagellin-transfected macrophages were incubated with *S.*Typhi (2×10^8 bacteria/ml) for 1 h at 37°C. Supernatants from uninfected macrophages were used as controls. Following incubation, bacterial suspensions were centrifuged at 13,000 rpm and filtered supernatants were analyzed for *S.*Typhi flagellin by immunoblotting with *S*.Typhi flagellin-specific monoclonal antibody as described previously (1). For detection of LPS, blots were stripped with a solution of low pH (0.15 M NaCl containing acetic acid, pH 3) and reprobed with monoclonal antibody to *S.*Typhi LPS as described previously (1, 2).

IL-1β ELISA

IL-1β levels in cell culture supernatants were measured using a commercially available ELISA kit (Opt EIA Mouse IL-1βSet; BD Pharmingen) according to the manufacturers' instructions.

β-galactosidase assay for *fliC* promoter activity

A *S*.Typhimurium strain containing the *fliC* promoter region fused to a promoterless lacZ (*S*.Tym *fliC*-lacZ) was used for assessing *fliC* promoter activity. $4X10^8$ *S*.Tym *fliC*-lacZ were treated for 30 min at 37ºC with 1 ml filter-sterilized supernatants from macrophages previously infected with WT *S*.Typhimurium. Bacterial suspensions were centrifuged at 13,000 rpm for 5 min. Pellets were resuspended in 0.5 ml PBS, sonicated, and sonicates were centrifuged at 13,000 rpm to remove debris. β-galactosidase activity in the supernatants was assayed by a colorimetric assay with chlorophenol red β-D-galactoside as the substrate and measuring absorbance at 570 nm.

Transfections

Endotoxin-free flagellin was purified from *S.*Typhimurium as described (4) or obtained commercially (Invivogen). Macrophages from C57BL/6 mice were seeded in 6-well plates and stimulated for 2 h with LPS (100 ng/ml). Cells were washed 2-3 times with serum-free medium and transfected with 200 ng or 1000 ng flagellin for 3 h using FuGENE 6 transfection reagent (Promega). Cell-free supernatants were analyzed for IL-1β, LDH and for their ability to trigger flagellin release from *S.*Typhi. LDH activity in supernatants was determined using the CytoTox 96 assay (Promega), and IL-1β was measured by ELISA.

Proteinase K treatment

Supernatants from *S.*Typhimurium–infected macrophages were digested with proteinase K (1 μg/ml) for 1 h at 37°C, followed by heat treatment at 80°C for 45 min to inactivate proteinase K. Untreated and proteinase K–treated supernatants were used to stimulate *S.*Typhi. Flagellin released into the supernatants was analyzed by immunoblotting.

Delipidification

Supernatant from *S.*Typhimurium–infected macrophages was extracted with a chloroform / methanol mixture (1:1). The lipid-free aqueous phase was collected, incubated with *S.*Typhi for 1 h and flagellin released by bacteria was analyzed by immunoblotting.

TLC and MS analysis

The mouse macrophage cell line, RAW 264.7, was cultured overnight in serum-free medium. Cells were infected with *S.*Typhimurium at a bacteria to cell ratio of 50:1 for 30 min. Cells were washed with medium containing gentamycin (100 μg/ml) to remove extracellular bacteria. Fresh gentamycin-containing medium was added to cells and supernatant collected after 1 h was processed for lipid extraction according to the procedure of Bligh and Dyer (5). Lipids were separated by TLC and spots were developed using iodine vapors. Lipids were extracted from the spots with a chloroform / methanol mixture (1:1) and analyzed by LCMS (Waters 1525, Micromass LCT).

Isolation of intracellular *Salmonella*

Ex vivo-differentiated BMDMs were infected with *S.*Typhimurium at a MOI of 25 for 30 min. Immortalized BMDMs or RAW 264.7 cells were infected at a MOI of 20 for 1 h. These MOIs were chosen so as to ensure lower cell death which enabled assessment of cells for flagellin expression by intracellular *Salmonella* over an extended period of infection. In some

experiments, WT BMDMs were pre-treated with iPLA2–specific inhibitor (FKGK-11) or cPLA2–specific inhibitor (Pyrrophenone) for 30 min prior to infection. Extracellular bacteria were removed by washing and macrophages were incubated in cell culture medium containing gentamycin (100 μg/ml). At different time points up to 48 h post infection, cells were washed to remove gentamycin and lysed in RPMI-1640 containing 0.2% Triton X-100. Intracellular bacteria were expanded in serum-free RPMI-1640 at 37°C with shaking at 200 rpm. Bacteria were washed and bacterial numbers were normalized across all samples. Flagellin expression was analyzed by immunoblot or qPCR.

Immunoblot analysis

For analysis of extracellular flagellin release, filter-sterilized supernatants from *S*.Typhimurium-infected macrophages were boiled in Laemelli buffer and run on a 12% SDS-polyacrylamide gel. Filter-sterilized supernatants from *S*.Typhi incubated with supernatants from *S*.Typhimurium-infected or flagellin-transfected macrophages were also run similarly. For analysis of flagellin expression by intracellular *Salmonella*, bacteria obtained from infected macrophages were enumerated spectrophotometrically by measuring absorbance at 630nm. Equal numbers of bacteria were boiled in Laemelli buffer and run on a 12% SDS-polyacrylamide gel (10^6 bacteria/lane). Proteins were transferred to nitrocellulose membranes. Flagellin, LPS and DnaK expression was analyzed by immunoblotting with specific antibodies. Reactive bands were visualized with Enhanced Chemiluminescence Reagent (Pierce).

For analysis of protein expression in macrophages, *ex vivo* differentiated BMDMs were seeded in a 6-well plate at a density of $2x10^6$ cells per well. Cells were infected with *Salmonella* at a MOI of 25 for 30 min. Cells were washed with RPMI to remove extracellular bacteria and incubated in RPMI containing 100 μg/ml gentamycin. At 1, 3, 5, 10 and 18 h post infection, cells were washed with RPMI and lysed in RIPA lysis buffer. Protein concentration in cell lysates was estimated colorimetrically using BCA protein assay kit (Pierce) and 40 μg cell lysate per sample was run on a 12% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membranes. Expression of NLRC4, iPLA2, NLRP3, ASC, caspase-1 and ERK2 (loading control) was analyzed by immunoblotting with specific antibodies.

Quantitative RT-PCR

For analysis of macrophage gene expression, cells were infected as described above. At different times post infection, cells were lysed with TRIzol reagent (Invitrogen) and RNA was extracted using RNeasy kit (Qiagen). Splenocytes harvested from infected or uninfected mice

on days 2, 3 and 5 were adherence-purified and incubated in medium containing 100 μg/ml gentamycin for 1 h followed by RNA extraction. cDNA was synthesized using Superscript III first strand kit, according to the instructions provided by the manufacturer (Invitrogen) and expression of various genes such as NLRC4, iPLA2, cPLA2, NLRP3, ASC, caspase-1, IFN-β and MX2 was determined by quantitative RT-PCR. Gene expression in each sample was normalized to that of β-actin which served as an internal control, and represented as fold change relative to that of uninfected cells i.e. cells incubated in media alone for the same time. For analysis of *fliC* gene expression by intracellular *Salmonella*, bacterial RNA was isolated as described previously (6) and cDNA was synthesized with random hexamers using RevertAid first strand cDNA synthesis kit (Thermo). qPCR analysis was performed using primers specific for *fliC* or *dnaK*. Levels of *fliC* mRNA were normalized to that of *dnaK* and fold change in *fliC* gene expression at different time points post infection was plotted relative to that of intracellular *Salmonella* at 6 h post infection.

Confocal microscopy

For analysis of flagellin expression by confocal microscopy, BMDMs were infected with *S*.Typhimurium *fliC*-GFP (*S*.Typhimurium 14028::pkas32::pfliC::gfp, kindly provided by Dr. Brad Cookson, University of Washington, Seattle) at a MOI of 25 for 30 min. Extracellular bacteria were removed by washing and macrophages were incubated in cell culture medium supplemented with gentamycin (100 μg/ml). At different time points post infection, macrophages were permeabilized on ice using chilled methanol for 5 min followed by staining for *Salmonella* LPS. Intracellular *Salmonella* were visualized as LPS-positive and scored for flagellin expression based on GFP positivity. The number of *fliC*-positive bacteria per condition was quantified as a fraction of the total number of LPS-positive bacteria imaged.

Estimation of intracellular LPC

BMDMs were lysed in lysis buffer containing NP-40 (10 mM Tris-HCl pH 7.2, 140 mM NaCl, 2 mM EDTA, 1% NP-40). LPC levels in the lysate were estimated using a competitive ELISA according to the manufacturer's instructions (MyBioSource Inc, Cat# MBS2031889). Protein concentration in cell lysates was estimated using BCA protein assay kit (Pierce). Levels of LPC were then normalized to that of total protein in the cell lysates.

In vivo experiments

S.Typhimurium grown to mid to late log phase (8-10h) in LB broth at 37°C with shaking at 220rpm were washed with serum-free RPMI and bacteria were enumerated spectrophotometrically by recording absorbance at 630 nm. WT, NLRC4$^{-/-}$, Caspase-1/11$^{-/-}$ and IFNAR$^{-/-}$ C57BL6 mice were infected i.p. with 200 CFU of *S*.Typhimurium. For experiments with the *in vivo* iPLA2 inhibitor, FKGK-18, mice were treated with inhibitor (20 mg/kg body weight i.p.) (7) or with vehicle (DMSO) in PBS daily starting from one day prior to infection until day 5 post infection. Mice were euthanized on days 2 and day 5 post infection and spleens were harvested. A single cell suspension of splenocytes was prepared, RBCs were lysed using Ack lysing buffer and macrophages were allowed to adhere to plastic in a tissue culture dish for 1 h in RPMI containing 5% serum and gentamycin (100 µg/ml). Non-adherent cells were removed by washing and adherent cells were lysed in RPMI-1640 containing 0.2% Triton X-100 for 5 min to harvest intracellular bacteria.

Quantification and Statistical Analysis

Statistical analysis

Student's *t*-test was used to calculate *p*-values. Data are expressed as mean ± SD (or as indicated in the figure legends). Statistical analyses were performed using Student's *t* test in Microsoft Excel. p-values less than 0.05 were considered statistically significant. Statistical parameters such as the value of n, the number of replicates, precision measures and statistical significance are reported in the figures and figure legends.

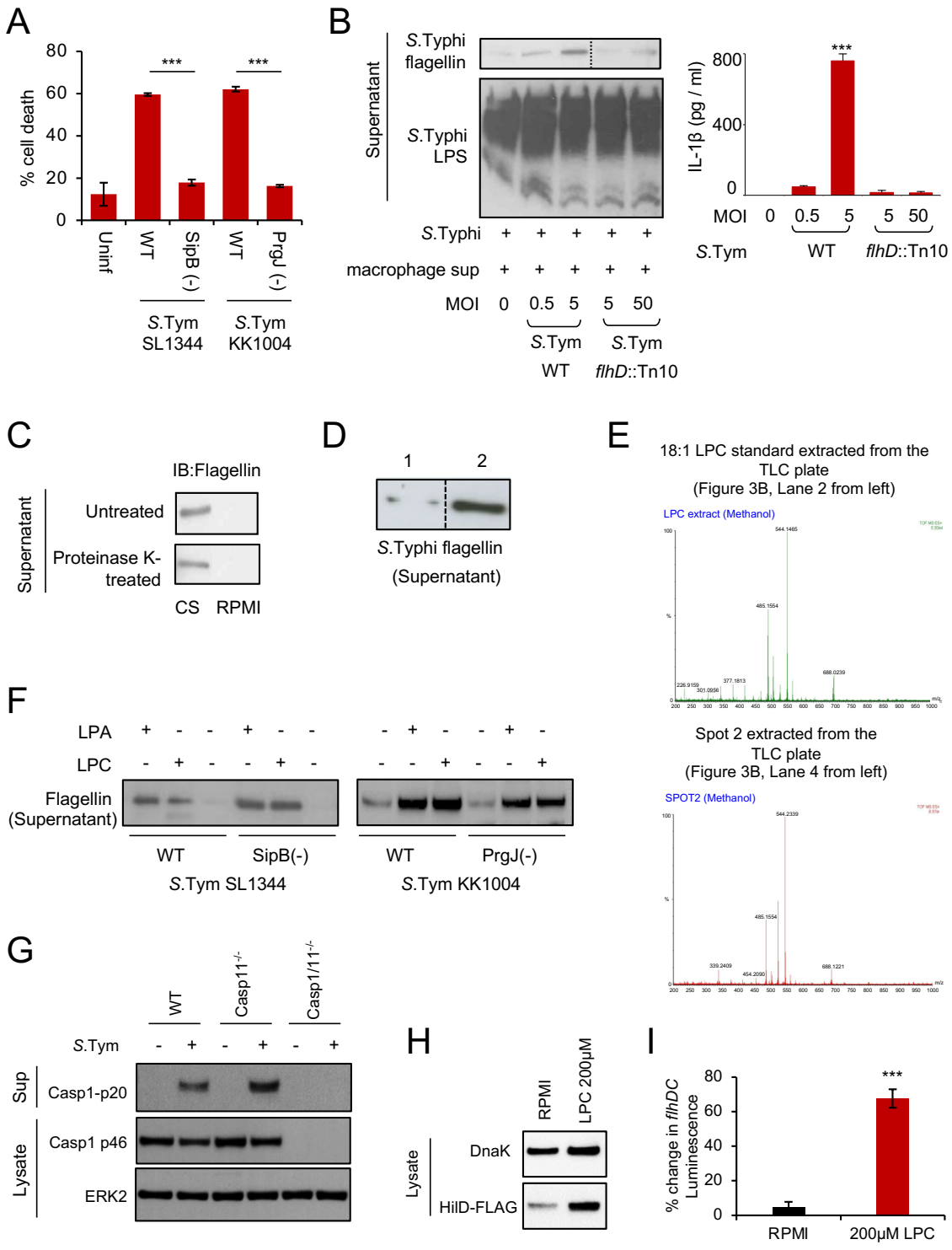
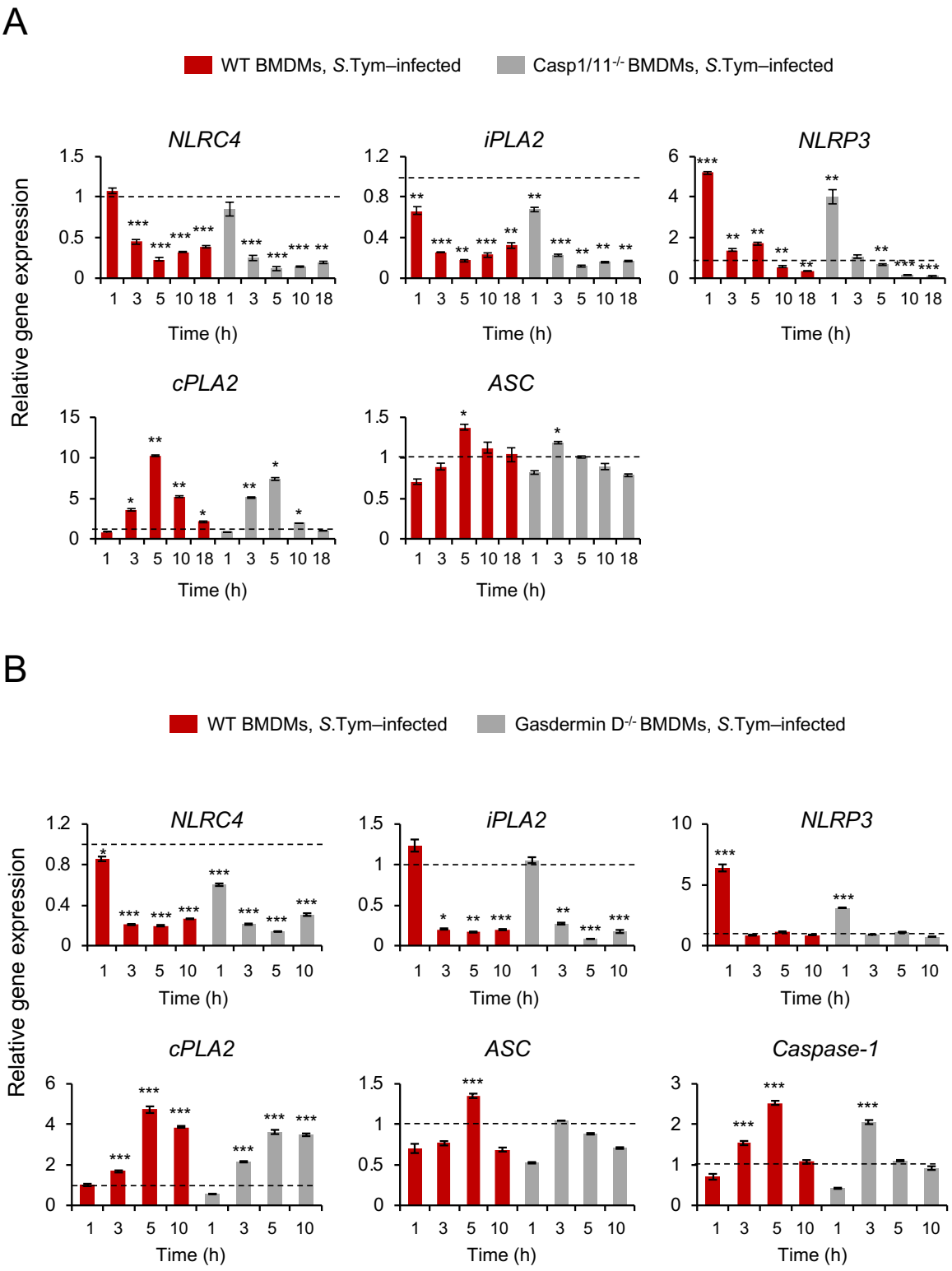


Fig. S1. Macrophage pyroptosis-derived lysophospholipids activate release of flagellin from *Salmonella*. Related to Figure 2 and Figure 3.

(**A**) Estimation of cell death by lactate dehydrogenase (LDH) release in filter-sterilized supernatants of peritoneal macrophages infected with 50 MOI of log phase WT *S*.Typhimurium SL1344 and its SipB-deficient derivative or WT *S*.Typhimurium KK1004 and its PrgJ-deficient derivative for 1 h. (**B**) Immunoblot for flagellin and LPS (left) released by *S*.Typhi in response to filter-sterilized supernatants of

S.Typhimurium-infected macrophages. LPS-primed peritoneal macrophages were infected with log phase WT (KK1004) or an aflagellar mutant (*flhD*::Tn10) of *S*.Typhimurium KK1004 at the indicated MOIs for 30 min. Extracellular bacteria were removed and cells were incubated in fresh medium for 2 h. IL-1β in supernatants was measured by ELISA (right), and filter-sterilized supernatants were analyzed for their ability to induce flagellin secretion from *S.*Typhi by immunoblot (left). (**C**) Immunoblot for flagellin released by *S.*Typhi in response to proteinase K-treated supernatant from *S*.Typhimurium–infected macrophages. Serum-free cell culture-supernatant from log phase *S*.Typhimurium-infected macrophages was treated with Proteinase K (1 µg/ml) for 1 h prior to incubation with *S.*Typhi. Flagellin secreted by *S.*Typhi was analyzed by immunoblot. (**D**) Immunoblot for flagellin released by *S*.Typhi in response to supernatants of *S*.Typhimurium-infected macrophages. Cell-free supernatant from *S*.Typhimurium–infected RAW 264.7 cells was incubated with *S*.Typhi and flagellin in the supernatant was analyzed by immunoblot. (**E**) Analysis of LPC in supernatant of *S*.Typhimurium-infected RAW 264.7 cells by LCMS. The product-ion spectra of the LPC standard (18:1 LPC) and the corresponding spot in *S*.Typhimurium-infected macrophage supernatant (Spot 2) in Figure 3B extracted from silica are shown. LPC was identified as an active component. (**F**) Immunoblot for flagellin in filter-sterilized supernatants of WT *S*.Typhimurium SL1344 and its SipB-deficient derivative or WT *S*.Typhimurium KK1004 and its PrgJ-deficient derivative stimulated with 200 µM lysophospholipids (LPA and LPC) for 1 h. (**G**) Immunoblot for caspase-1 activation in macrophages infected with *S*.Typhimurium. Peritoneal macrophages from WT, Caspase-11$^{-/-}$ and Caspase-1/11$^{-/-}$ mice were infected with 20 MOI of log phase *S*.Typhimurium for 30 min. Caspase-1 p20 in the supernatant and Procaspase-1 (p46) in the lysate was assayed by immunoblotting. ERK2 was used as a loading control. (**H**) Immunoblot showing increased expression of HilD upon treatment with LPC. A reporter strain of *S*.Typhimurium 14028 expressing FLAG-tagged HilD (8) was grown to log phase followed by treatment with 200 µM LPC or RPMI as a control for 30 min. Expression of HilD was assessed by immunoblotting. DnaK served as a loading control. (**I**) Luciferase reporter assay showing increased *flhDC* promoter activity upon treatment with LPC. A reporter strain of *S*.Typhimurium 14028 harboring transcriptional fusion of the *flhDC* promoter region to the *luxCDBAE* luciferase operon of *Photorhabdus luminescens* (P_{flhDC}-luxCDABE) (8) was grown to log phase followed by treatment with 200 µM LPC or RPMI as a control for 30 min. Luminescence was measured using a plate reader and normalized for cell density by recording absorbance at 595 nm. % increase in luciferase activity upon LPC treatment was determined relative to that of cells treated with RPMI as a control. Data in A-D and F are representative of three independent experiments. Data in E and G-I are representative of two independent experiments. Error bars on graphs are mean±SD of triplicates. ** $p<0.01$, *** $p<0.001$.



310

Fig. S2. Repression of NLRC4 and iPLA2 gene expression in *Salmonella*-infected macrophages is independent of pyroptosis. Related to Figure 4.

(A, B) Quantitative RT-PCR for the indicated genes in infected BMDMs showing that *Salmonella* infection downregulates NLRC4 and iPLA2 in Caspase-1/11$^{-/-}$ BMDMs (**A**) and Gasdermin D$^{-/-}$ BMDMs (**B**) similar

to WT BMDMs. Gene expression at each time point was normalized to the uninfected control at that time point (dashed line, set to 1) as described in Figure 4B. Data are representative of two independent experiments. Error bars on graphs are mean±SD of triplicates. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

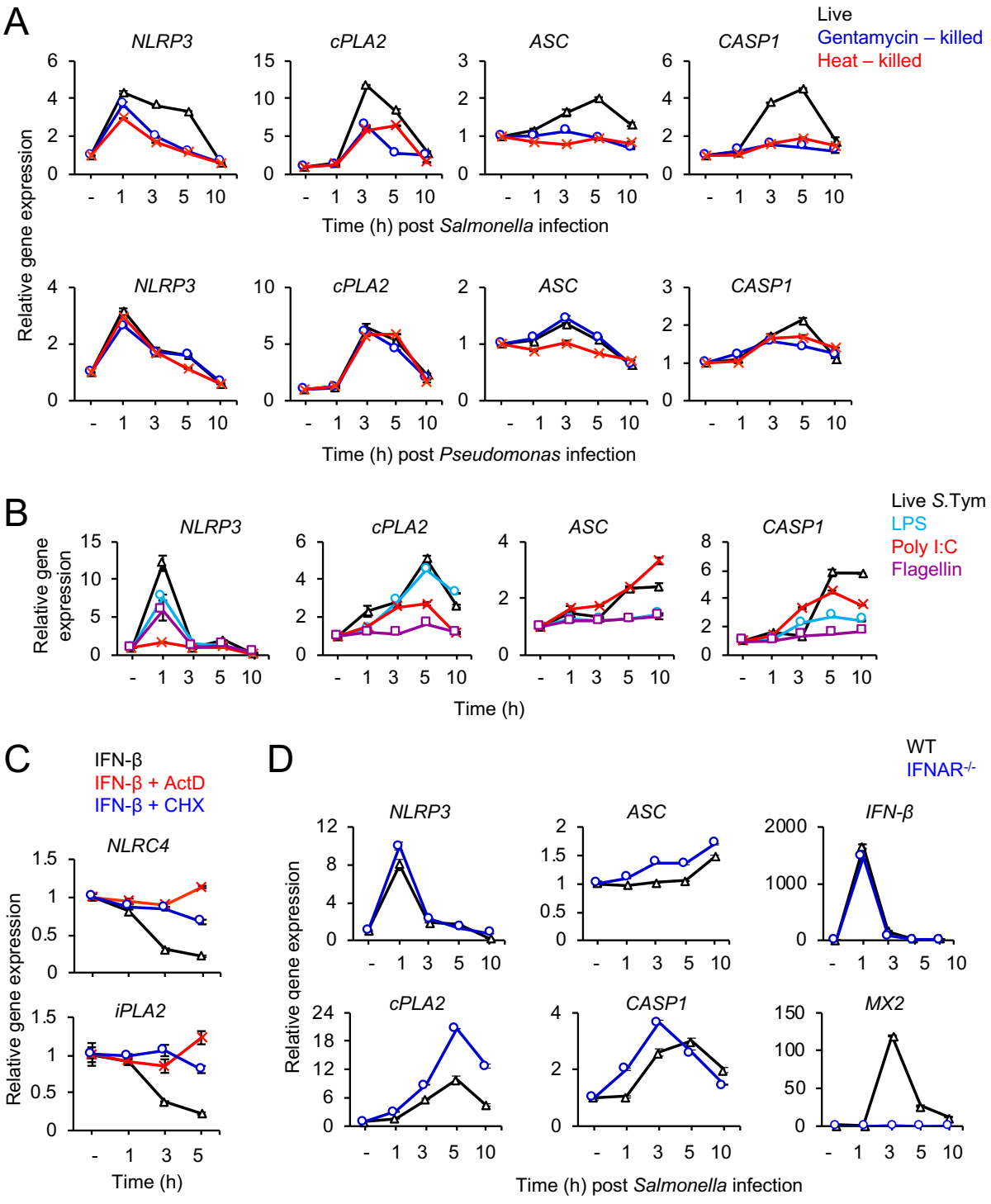


Fig. S3. Type 1 IFN signaling represses NLRC4 and iPLA2 expression. Related to Figure 5. (**A**) Quantitative RT-PCR for the indicated genes in infected BMDMs. WT BMDMs were infected with live or killed *S.*Typhimurium (upper panel) or *Pseudomonas aeruginosa* (lower panel) as described in Figure 5A followed by gentamycin protection for varying times. RNA was extracted from BMDMs and expression of the indicated genes was analyzed by qPCR. (**B**) Quantitative RT-PCR for the indicated genes in *S.*Typhimurium-infected BMDMs or BMDMs activated with TLR ligands. WT BMDMs were infected with

log phase *S*.Typhimurium as in Figure 5A followed by gentamycin protection or treated with TLR agonists LPS (1 μg/ml), flagellin (1 μg/ml) or poly I:C (5 μg/ml) for the indicated times. (**C**) Quantitative RT-PCR for NLRC4 and iPLA2 in BMDMs stimulated with IFN-β. WT BMDMs were either left untreated or treated with Actinomycin D, an inhibitor of *de novo* transcription (10 μg/ml) or Cycloheximide, an inhibitor of *de novo* translation (100 μg/ml) for 30 min. Cells were then treated with IFN-β (1000 U/ml) for designated times. Expression of NLRC4 and iPLA2 was analyzed by qPCR. (**D**) Quantitative RT-PCR for the indicated genes in infected WT and IFNAR$^{-/-}$ BMDMs. BMDMs were infected with 25 MOI of log phase *S*.Typhimurium for 30 min as described in Figure 5A followed by gentamycin protection for the designated times. Expression of the indicated genes was analyzed by qPCR. A and C show one representative of three independent experiments. Data in B and D are one representative of two independent experiments. Error bars are mean±SD of triplicates.

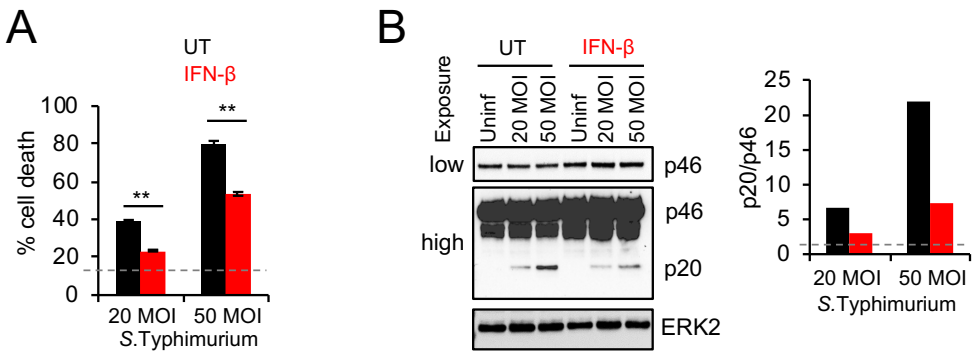
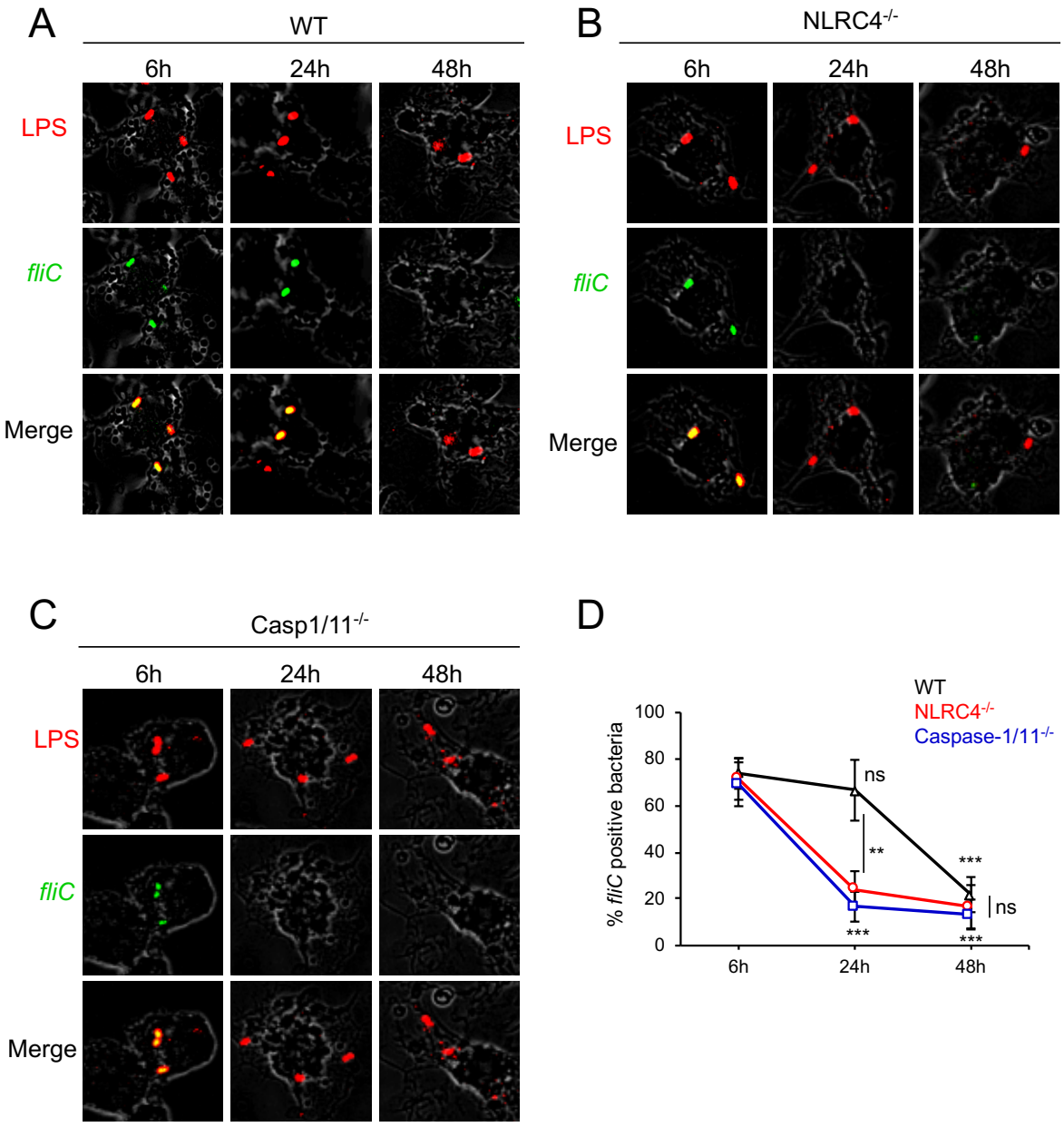


Fig. S4. IFN-β treatment reduces *Salmonella*-induced macrophage pyroptosis. Related to Figure 5. (**A**) Estimation of cell death in WT BMDMs pre-treated with IFN-β. WT BMDMs were pre-treated with 1000 U/ml of purified IFN-β for 10 h followed by infection for with 20 and 50 MOI of log phase *S*.Typhimurium for 30 min. LDH release in the supernatant was measured. Basal LDH release by uninfected BMDMs is represented by the dashed line. (**B**) Immunoblot (left) and quantification (right) of caspase-1 activation in WT BMDMs pre-treated with IFN-β. WT BMDMs were pre-treated with purified IFN-β followed by infection with *S*.Typhimurium as in (A). Total cell lysate + supernatant was subjected to western blot analysis. Band intensities were normalized to that of ERK2 using ImageJ and Caspase-1 p20 band density was plotted relative to that of total Procaspase-1 (p46). Density unit of uninfected BMDMs is represented by the dashed line. Data are representative of two independent experiments. Error bars on graphs are mean±SD of triplicates. ** $p<0.01$.



355 **Fig. S5. Kinetics of flagellin downregulation by *Salmonella in situ* in NLRC4$^{-/-}$ and Caspase-1/11$^{-/-}$ BMDMs.** Related to Figure 6.

(**A-D**) Representative confocal immunofluorescence images (A-C) and quantification (D) showing *fliC* expression by *S*.Typhimurium in macrophages of the indicated genotypes. BMDMs of the indicated genotypes were infected with log phase *fliC*-GFP reporter strain of *S*.Typhimurium 14028 at a MOI of 25
360 for 30 min. Extracellular bacteria were removed and cells were incubated for the indicated times in complete medium containing gentamycin (100 µg/ml). At indicated time points post infection, cells were stained with antibody to *S*.Typhimurium LPS. Intracellular *Salmonella* were visualized as LPS-positive (red), flagellin-positive (green) and double positive (yellow). The percentage of *fliC*-positive bacteria per condition were enumerated as a fraction of total LPS-positive bacteria. At least 25 cells were imaged per
365 condition. Data are representative of two independent experiments. Error bars on graphs are mean±SEM. ** p<0.01, *** p<0.001. ns: not significant.

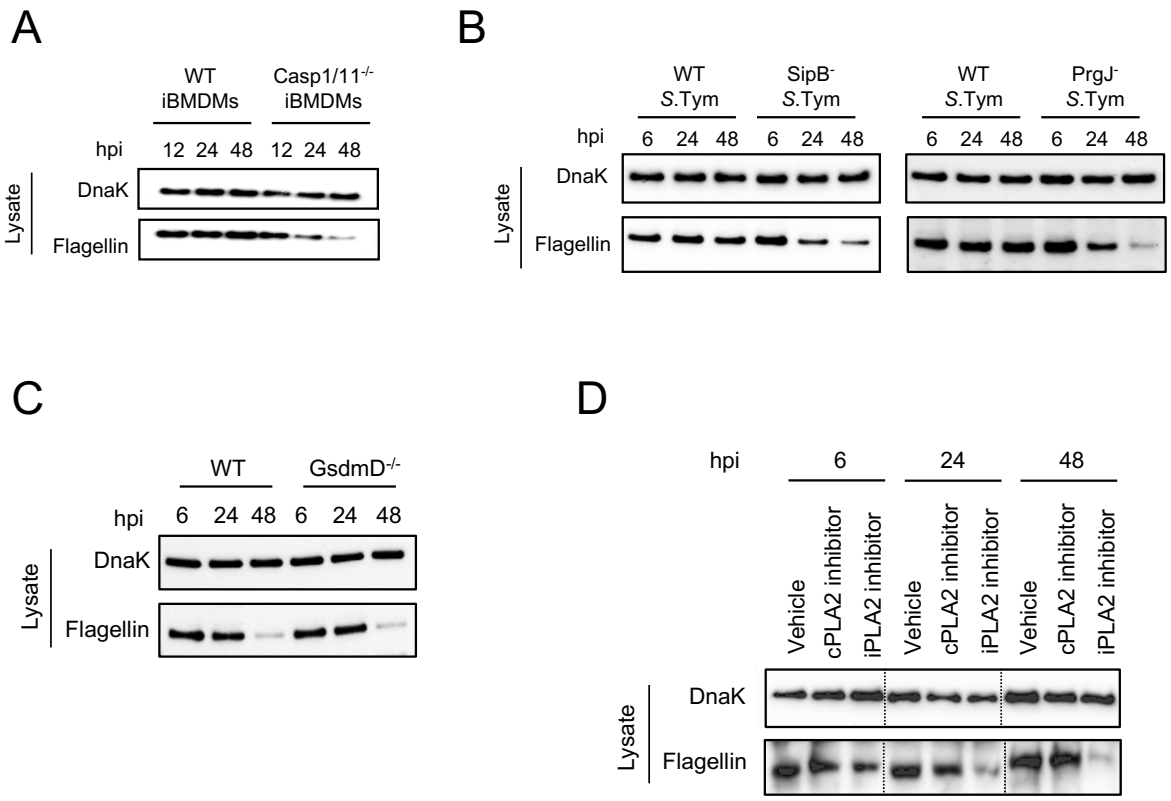
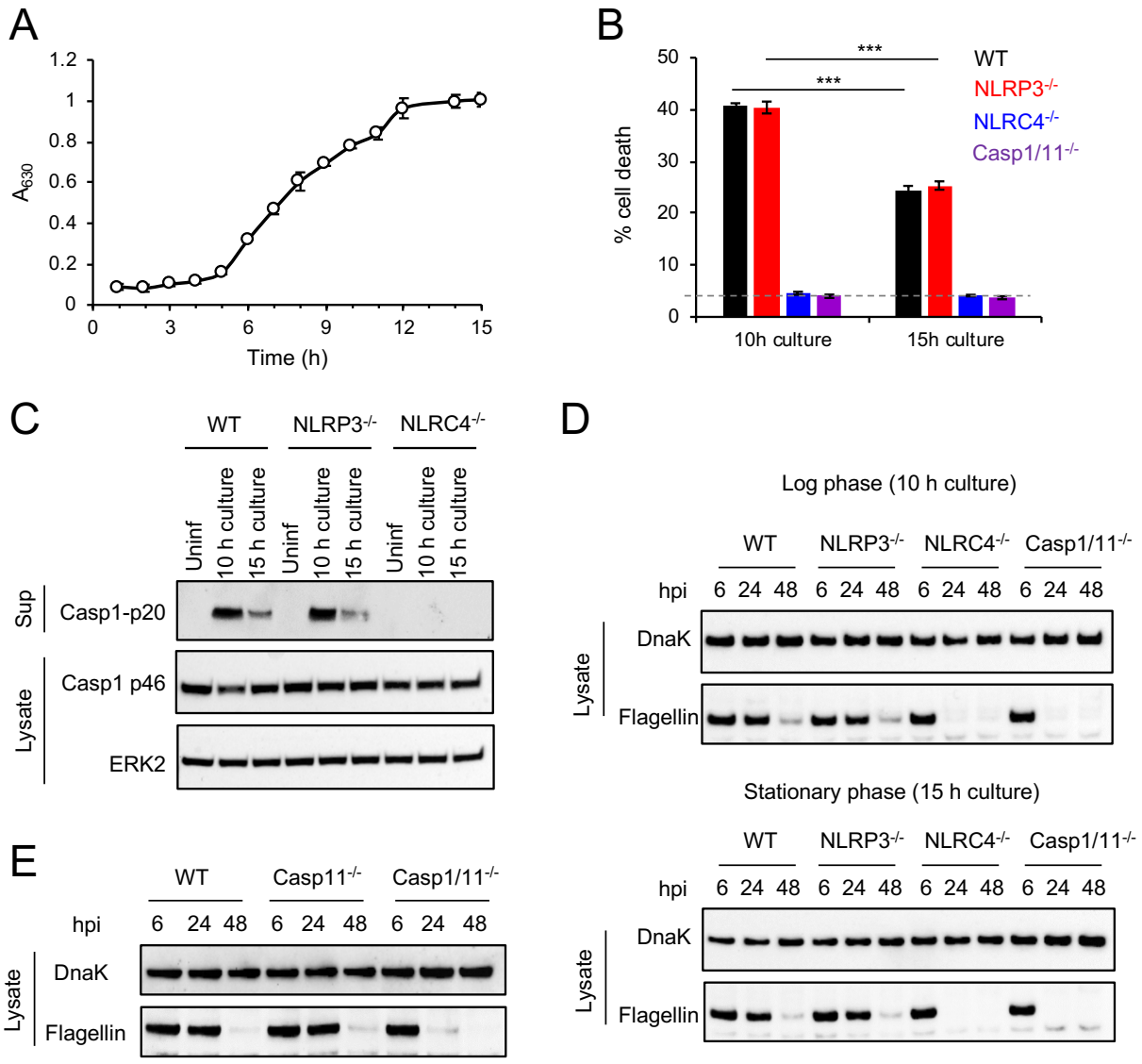


Fig. S6. Caspase-1 and iPLA2 activity controls expression of flagellin by intracellular *Salmonella*.
370 Related to Figure 6.
(**A**) Immunoblot for flagellin and DnaK expression by intracellular *Salmonella* harvested from immortalized BMDMs (iBMDMs) of the indicated genotypes showing faster downregulation of flagellin by bacteria

recovered from caspase1/11$^{-/-}$ BMDMs compared to those recovered from WT BMDMs. WT and caspase-1/11$^{-/-}$ iBMDMs were infected with *S*.Typhimurium at a MOI of 20 for 30 min. Extracellular bacteria were removed and cells were incubated for the indicated times in complete medium containing gentamycin (100 µg/ml). Intracellular bacteria were harvested and flagellin and DnaK were analyzed by immunoblot. hpi: hours post infection. (**B**) Immunoblot for flagellin and DnaK expression by intracellular *Salmonella* isolated from iBMDMs showing faster downregulation of flagellin by SipB–deficient or PrgJ–deficient bacteria compared to WT bacteria. iBMDMs were infected with log phase WT *S*.Typhimurium SL1344 or its SipB-deficient derivative and WT *S*.Typhimurium KK1004 or its PrgJ-deficient derivative as in (A). (**C**) Immunoblot for flagellin and DnaK expression by intracellular *Salmonella* recovered from WT and Gasdermin D$^{-/-}$ BMDMs. Cells were infected with 25 MOI of log phase *S*.Typhimurium for 30 min followed by gentamycin protection for the indicated times. Flagellin and DnaK expression by intracellular bacteria were assessed by immunoblot. (**D**) Immunoblot for flagellin and DnaK expressed by intracellular *Salmonella* in iBMDMs treated with PLA2 inhibitors or vehicle. iBMDMs were treated with inhibitors of cPLA2 or iPLA2 followed by infection with *Salmonella* as in Figure 6D. At 6, 24 and 48 h post infection, intracellular *Salmonella* were recovered and flagellin and DnaK expression was analyzed by immunoblot. (**E**) Observed outcomes of flagellin expression by intracellular bacteria in NLRC4 and Caspase-1/11$^{-/-}$ genotypes are not explained by selection bias or pyroptosis. Data are representative of two (B and C) or three (A and D) independent experiments.



395 **Fig. S7. Downregulation of flagellin expression by intracellular *Salmonella* is independent of growth phase of infecting *Salmonella*, NLRP3 and Caspase-11.** Related to Figure 6.
(**A**) Growth curve analysis of *S*.Typhimurium. *S*.Typhimurium were grown in LB broth at 37°C with shaking at 220rpm and absorbance was measured at 630nm at the indicated time intervals. Bacteria were in mid to late log phase after 8-10 h and in stationary phase after 15 h of growth in LB. (**B**) Effect of
400 growth phase of *Salmonella* on cell death in WT BMDMs. WT BMDMs were infected with 20 MOI of log phase (10 h culture) or stationary phase (15 h culture) *S*.Typhimurium for 30 min. LDH release in the supernatant was measured. Basal LDH release by uninfected BMDMs is represented by the dashed line. (**C**) Effect of growth phase of *Salmonella* on caspase-1 activation. WT BMDMs were infected as in (B). Caspase-1 p20 in the supernatant and Procaspase-1 (p46) in the lysate was assayed by immunoblotting.
405 ERK2 was used as a loading control. (**D**) Immunoblot for flagellin and DnaK expression by intracellular *Salmonella* showing similar kinetics of flagellin downregulation irrespective of the growth phase of infecting *Salmonella*. WT, NLRP3$^{-/-}$, NLRC4$^{-/-}$ and Caspase-1/11$^{-/-}$ BMDMs were infected with 20 MOI of log phase (10 h culture) or stationary phase (15 h culture) *S*.Typhimurium for 30 min followed by

gentamycin protection for the indicated times. Flagellin and DnaK expression by intracellular bacteria were assessed by immunoblot. (**E**) Immunoblot for flagellin and DnaK expression by intracellular *Salmonella* harvested at indicated time points showing similar kinetics of flagellin downregulation in WT and Caspase-11$^{-/-}$ BMDMs and faster downregulation in Caspase-1/11$^{-/-}$ BMDMs. BMDMs were infected as in (D). hpi: hours post infection. Data are representative of two independent experiments. Error bars on graphs are mean±SD of triplicates. *** $p<0.001$.

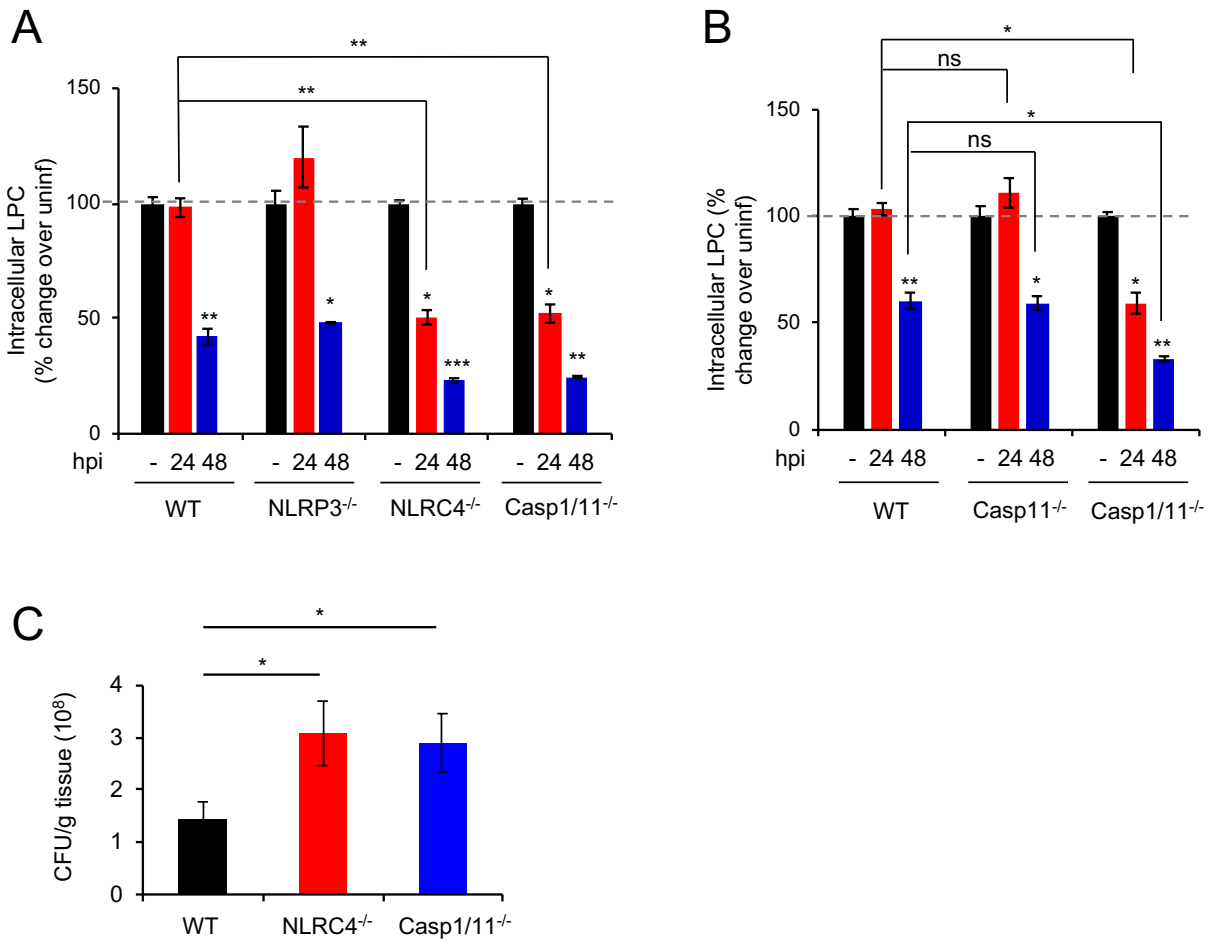


Fig. S8. NLRC4/Caspase-1 axis sustains intracellular levels of LPC in infected BMDMs. Related to Figure 6.

(A, B) Estimation of intracellular LPC in *Salmonella*-infected BMDMs. BMDMs of the indicated genotypes were infected with 20 MOI of log phase *S*.Typhimurium for 30 min followed by gentamycin protection for the indicated times. LPC concentration in cell lysates was measured by ELISA. The level of LPC in uninfected BMDMs of each genotype was set at 100% (dashed line) and LPC levels post infection are represented as % change in LPC compared to uninfected BMDMs of the respective genotype. hpi: hours post infection. (**C**) Bacterial load in spleens of infected WT, NLRC4$^{-/-}$ and Caspase-1/11$^{-/-}$ mice. WT, NLRC4$^{-/-}$ and Caspase-1/11$^{-/-}$ mice were infected with 200 CFU of log phase *S*.Typhimurium i.p. On day 5 post infection, spleens were homogenized and serial dilutions were plated onto *Salmonella–Shigella* (SS) agar. Bacterial colonies were enumerated and expressed as total colony forming units (CFU) per gram of the tissue. n=4 mice per group. Data are representative of two independent experiments. Error bars on graphs are mean±SD of triplicates. * p<0.05, ** p<0.01, *** p<0.001. ns: not significant.

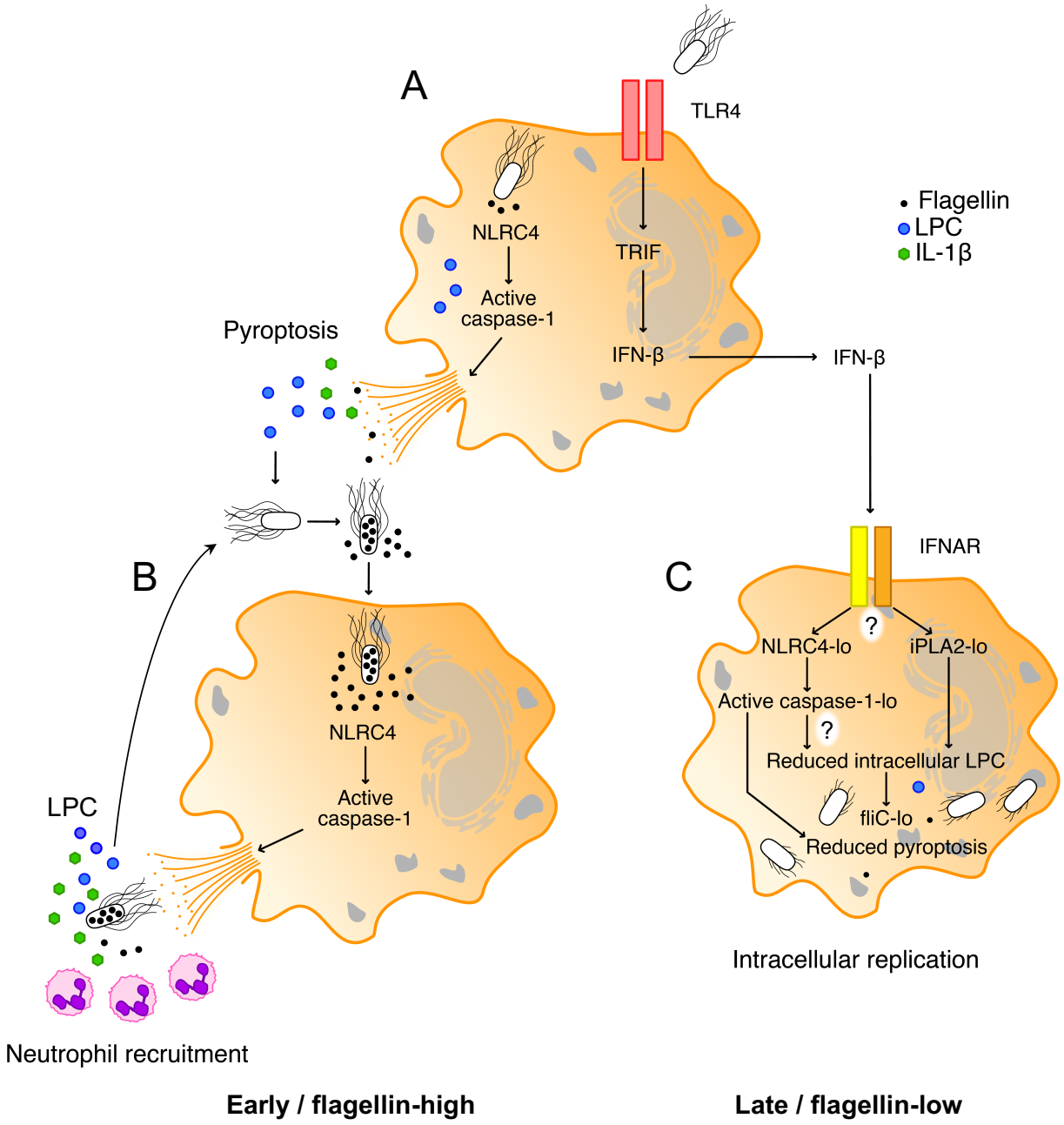


Fig. S9. Proposed model of NLRC4, iPLA2 and IFNAR-dependent switching of *Salmonella* from a flagellin-high to a flagellin-low phenotype inside macrophages.

(**A**) Initial infection of macrophages with *Salmonella* leads to robust early activation of caspase-1 through the NLRC4 inflammasome. Caspase-1 activation leads to pyroptosis with concomitant release of lysophospholipids such as LPC. LPC in turn promotes synthesis and release of flagellin from extracellular *Salmonella* (9). In addition, activation of TRIF likely by *Salmonella* LPS, leads to secretion of IFN-β from macrophages.

(**B**) Increased flagellin expression by extracellular bacteria increases their ability to induce pyroptosis upon internalization by healthy macrophages, which leads to increased activation of IL-1β and further release of LPC. The released LPC can in turn trigger *de novo* synthesis and release of flagellin from

extracellular bacteria, which sets up a feedforward, auto-amplifying circuit. In this manner, a flagellin^{hi} phenotype makes the pathogen more vulnerable to innate immune detection eventually leading to its clearance by neutrophils (10).

(**C**) IFNAR activation by IFN-β downregulates expression of NLRC4 and iPLA2 in the surviving macrophage population. This eventually creates an intracellular state that is depleted of lysophospholipids and refractory to NLRC4-dependent caspase-1 activation and pyroptosis. *Salmonella* adapts to this LPC-low intracellular environment and downregulates flagellin expression thereby transforming the bacterium to a flagellin^{lo} phenotype inside macrophages. The exact mechanisms by which IFNAR signaling downregulates NLRC4 and iPLA2 expression, and caspase-1 controls intracellular LPC levels are unknown.

Table S1. List of strains used in this study*

Strain	Description	Origin	Used in Figs:
SL1344	*S*.Typhimurium SL1344 WT	E. Charpentier (11)	All unless specified otherwise
EC428 (SB169)	*sipB*⁻ *S*.Typhimurium SL1344 *sipB::aphT*-Km^R	E. Charpentier (12)	2D, S1A, S1F, S6B
KK1004	*S*.Typhimurium strain LT2 *fljB*^{enx} *vh2* phase 1 locked derivative of LT2	K. Kutsukake (13)	2A, 2D, S1A, S1B, S1F, S6B
KK1110	LT2 *fliC*:: Mu*d1*(Amp^R *lac*) *fljB*^{enx} *vh2* (KK1004 *fliC*-lac operon fusion)	K. Kutsukake (14)	2C
KK2040	LT2 *flhD3425*::Tn*10 fljB*^{enx} *vh2*	K. Kutsukake (13)	S1B
TH25748	LT2 *zec-7080*::Tn*10d*Tc Δ*prgJ60 fljB*^{enx} *vh2*	This study (K. Hughes)	2D, S1A, S1F, S6B
TH2949	LT2 *fliD68*::Tn*10 fljB*^{enx} *vh2*	This study (K. Hughes)	2A
BC1091	ST14028 *fliC*^{WT}/pKAS32::*fliC::gfp* (ST14028 *fliC*-GFP)	B. Cookson (15)	1D, S5A-D
TH25823	ST14028 *zec-7080*::Tn*10d*Tc hilD227::3xFLAG(HilD-FLAG) (ST14028-*hilD227*-3xFLAG)	This study (K. Hughes)	S1H
TH25679	ST14028 *zec-7080::*Tn*10d*Tc *P*$_{flhDC}$8093(P$_{flhDC}$-*luxCDABE*-Km-P$_{flhDC}$$^+$) (ST14028 *flhDC*-luciferase)	This study (K. Hughes)	S1I
S.Typhi	*S*.Typhi Vi-negative clinical isolate	G. Mehta (16)	2E, 3A, S1B, S1C, S1D

* The LT2 strains include a deletion of the *fljBA* operon and *hin* recombinase that is a flagellar phase 1 locked (FliC^{ON}) background designated *fljB*^{enx} *vh2* (17), which was found to be deleted for the phase 2 flagellar region (Δ(*hin-fljBA*)) (18). The Δ*prgJ60* allele is an in-frame nonpolar deletion of *prgJ* described previously (19). The *P*$_{flhDC}$8093(P$_{flhDC}$-*luxCDABE*-Km-P$_{flhDC}$$^+$ allele is a duplication of the *flhDC* promoter region with one copy controlling wild-type *flhDC* operon expression and the other controlling lux operon expression and constructed as described previously (8). The construction of *hilD227*::3xFLAG(HilD-FLAG) allele is described (8).

SI References

1. A. Qadri, S. Ghosh, S. Upadhyay, G. P. Talwar, Monoclonal antibodies against flagellar antigen of Salmonella typhi. *Hybridoma* **8**, 353-360 (1989).
2. A. Qadri, S. K. Gupta, G. P. Talwar, Monoclonal antibodies delineate multiple epitopes on the O antigens of Salmonella typhi lipopolysaccharide. *J Clin Microbiol* **26**, 2292-2296 (1988).
3. A. H. Fortier, L. A. Falk, Isolation of murine macrophages. *Curr Protoc Immunol* **Chapter 14**, Unit 14 11 (2001).
4. K. D. Smith *et al.*, Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial motility. *Nature immunology* **4**, 1247-1253 (2003).
5. E. G. Bligh, W. J. Dyer, A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* **37**, 911-917 (1959).
6. R. J. Nichols *et al.*, Phenotypic landscape of a bacterial cell. *Cell* **144**, 143-156 (2011).

7. R. N. Bone *et al.*, Inhibition of Ca2+-independent phospholipase A2beta (iPLA2beta) ameliorates islet infiltration and incidence of diabetes in NOD mice. *Diabetes* **64**, 541-554 (2015).
8. C. Mouslim, K. T. Hughes, The effect of cell growth phase on the regulatory cross-talk between flagellar and Spi1 virulence gene expression. *PLoS Pathog* **10**, e1003987 (2014).
9. N. Subramanian, A. Qadri, Lysophospholipid sensing triggers secretion of flagellin from pathogenic salmonella. *Nature immunology* **7**, 583-589 (2006).
10. E. A. Miao *et al.*, Caspase-1-induced pyroptosis is an innate immune effector mechanism against intracellular bacteria. *Nature immunology* **11**, 1136-1142 (2010).
11. S. K. Hoiseth, B. A. Stocker, Aromatic-dependent Salmonella typhimurium are non-virulent and effective as live vaccines. *Nature* **291**, 238-239 (1981).
12. K. Kaniga, S. Tucker, D. Trollinger, J. E. Galan, Homologs of the Shigella IpaB and IpaC invasins are required for Salmonella typhimurium entry into cultured epithelial cells. *J Bacteriol* **177**, 3965-3971 (1995).
13. K. Kutsukake, Y. Ohya, S. Yamaguchi, T. Iino, Operon structure of flagellar genes in Salmonella typhimurium. *Mol Gen Genet* **214**, 11-15 (1988).
14. K. Kutsukake, T. Iino, Role of the FliA-FlgM regulatory system on the transcriptional control of the flagellar regulon and flagellar formation in Salmonella typhimurium. *J Bacteriol* **176**, 3598-3605 (1994).
15. L. A. Cummings, W. D. Wilkerson, T. Bergsbaken, B. T. Cookson, In vivo, fliC expression by Salmonella enterica serovar Typhimurium is heterogeneous, regulated by ClpX, and anatomically restricted. *Mol Microbiol* **61**, 795-809 (2006).
16. A. Sharma, A. Qadri, Vi polysaccharide of Salmonella typhi targets the prohibitin family of molecules in intestinal epithelial cells and suppresses early inflammatory responses. *Proc Natl Acad Sci U S A* **101**, 17492-17497 (2004).
17. T. Iino, A Stabilizer of Antigenic Phases in Salmonella Abortus-Equi. *Genetics* **46**, 1465-1469 (1961).
18. K. Kutsukake, H. Nakashima, A. Tominaga, T. Abo, Two DNA invertases contribute to flagellar phase variation in Salmonella enterica serovar Typhimurium strain LT2. *J Bacteriol* **188**, 950-957 (2006).
19. D. H. Wee, K. T. Hughes, Molecular ruler determines needle length for the Salmonella Spi-1 injectisome. *Proc Natl Acad Sci U S A* **112**, 4098-4103 (2015).