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

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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.

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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

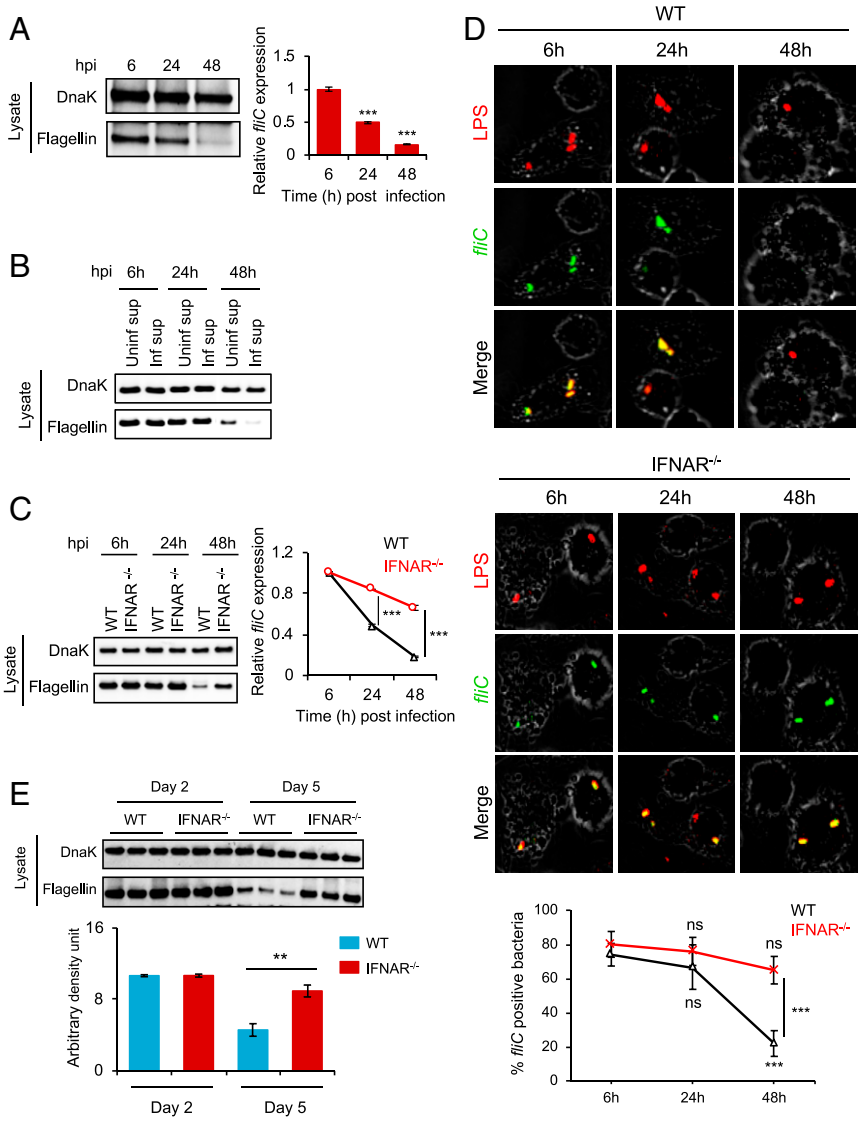

Fig. 1. IFNAR signaling promotes down-regulation of flagellin by *Salmonella*. (*A*) Immunoblot (*Left*) and qRT-PCR (*Right*) for flagellin expression by intracellular *Salmonella* obtained from infected mCSF-differentiated BMDMs at the indicated times. WT BMDMs were infected with 25 MOI of log phase *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

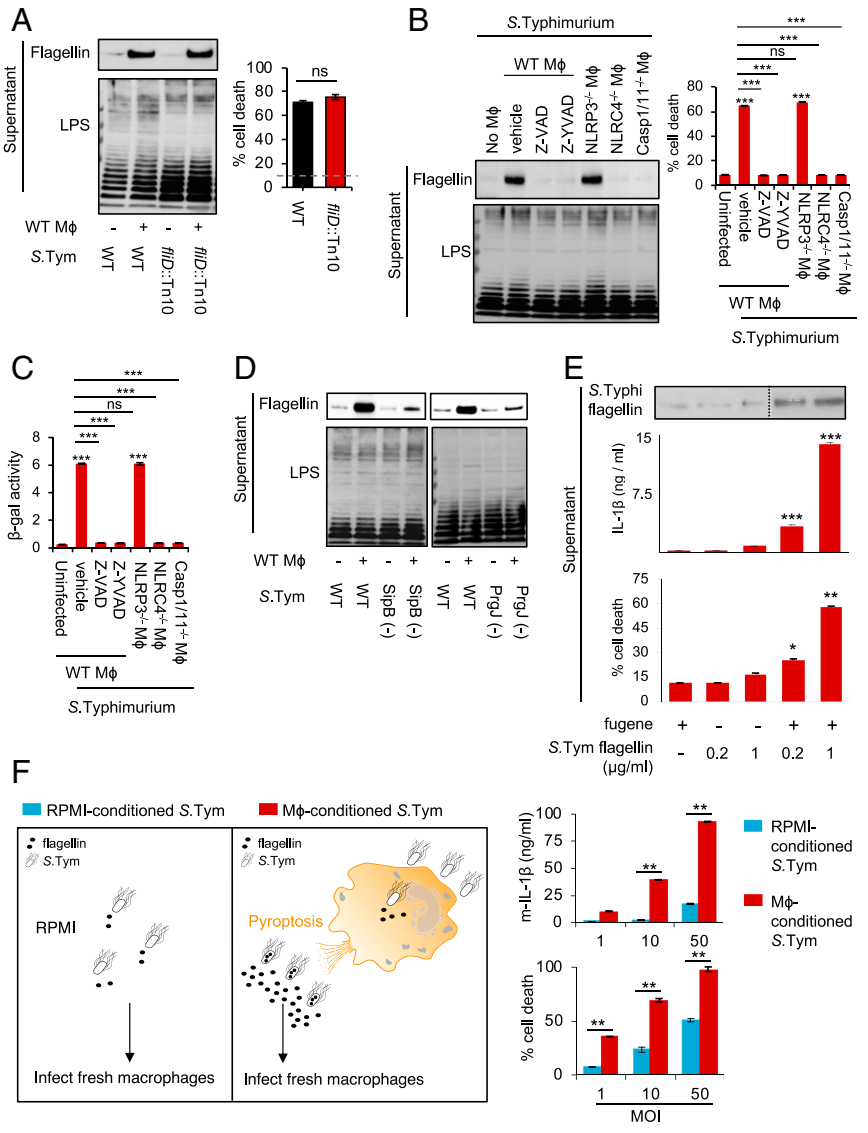

Fig. 2. NLRC4 inflammasome-mediated pyroptosis promotes increase in flagellin production by *Salmonella*. (*A*) Immunoblot for flagellin and LPS (*Left*) and assay for LDH (*Right*) in filter-sterilized supernatants of peritoneal macrophages infected with log phase WT *S*. Typhimurium KK1004 or its congenic 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

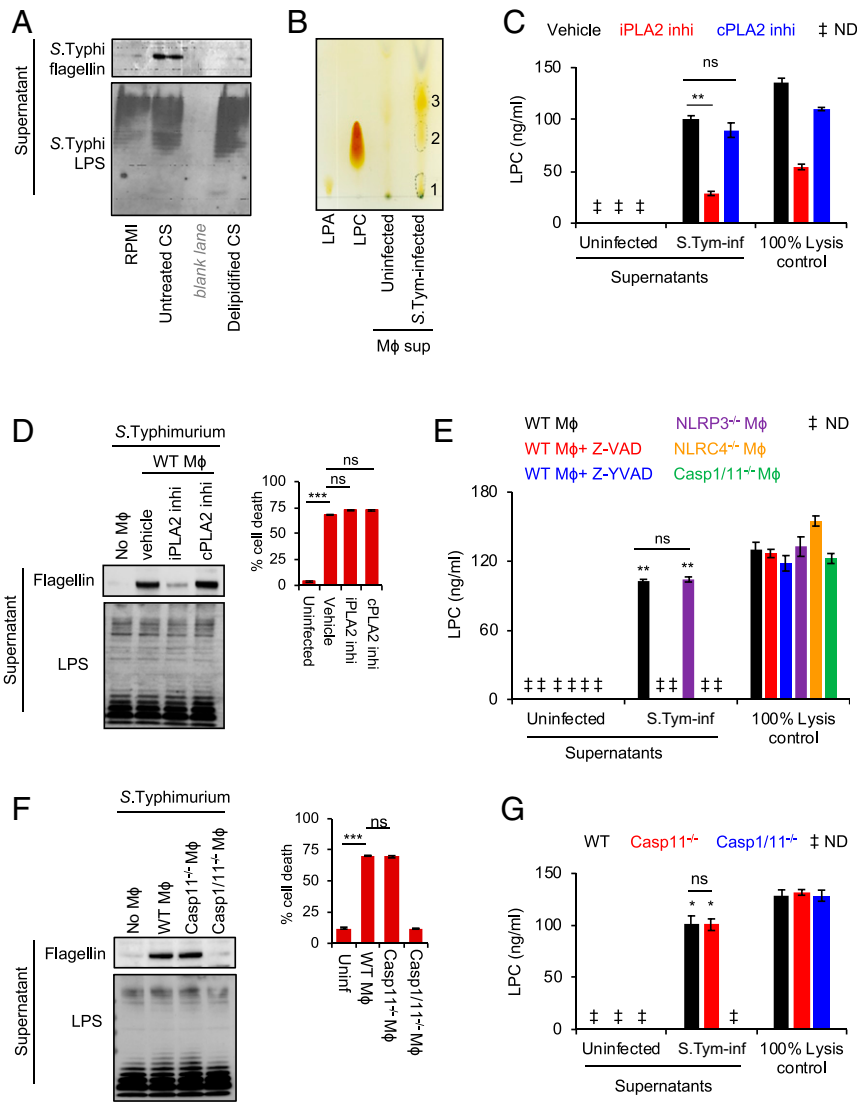

Fig. 3. Pyroptosis-derived lysophospholipids amplify production of flagellin by *Salmonella*. (*A*) Immunoblot for flagellin and LPS released by *S*. Typhi in response to delipidified supernatant from *S*. Typhimurium-infected macrophages. Serum-free cell culture supernatant from log phase *S*. Typhimurium-infected 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

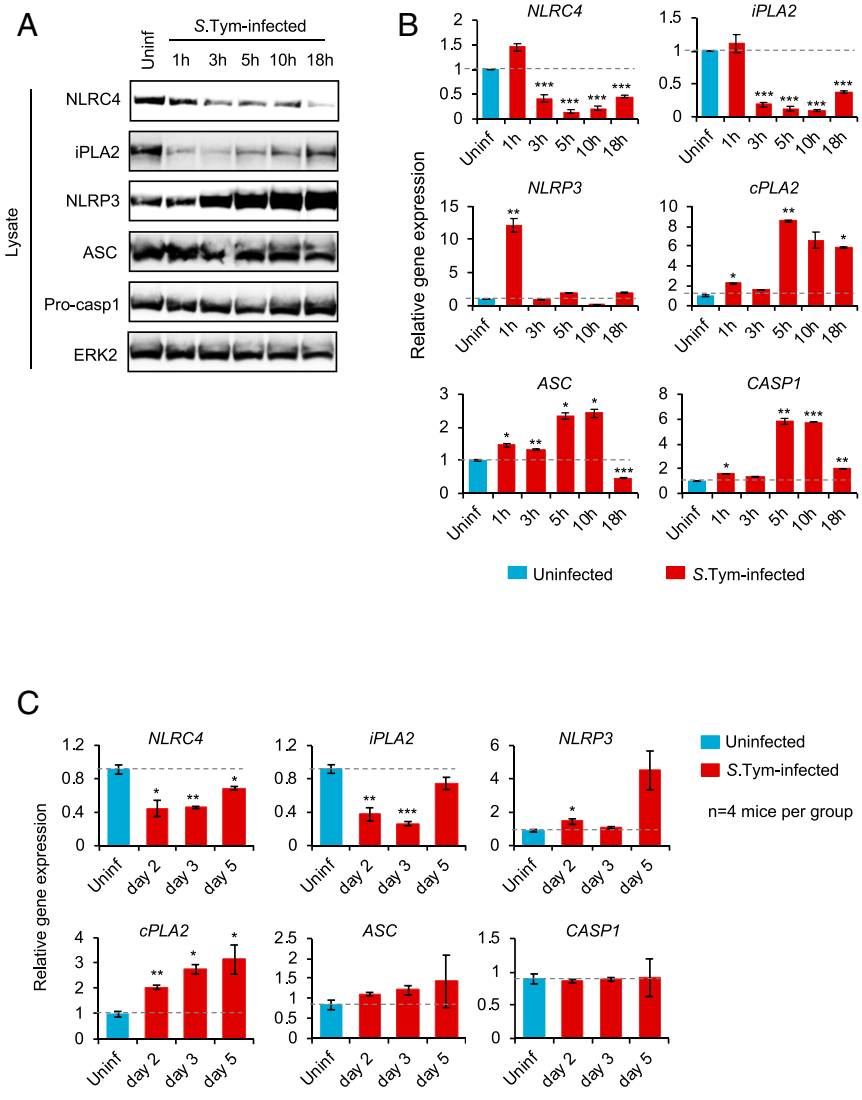

Fig. 4. Kinetics of NLRC4 and iPLA2 down-regulation by infected macrophages. (*A*) Immunoblot for inflammasome components and iPLA2 in lysates of uninfected BMDMs or BMDMs infected with log phase *S*. Typhimurium (25 MOI for 30 min), followed by gentamicin protection (100 μg/mL) for the indicated times. (*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

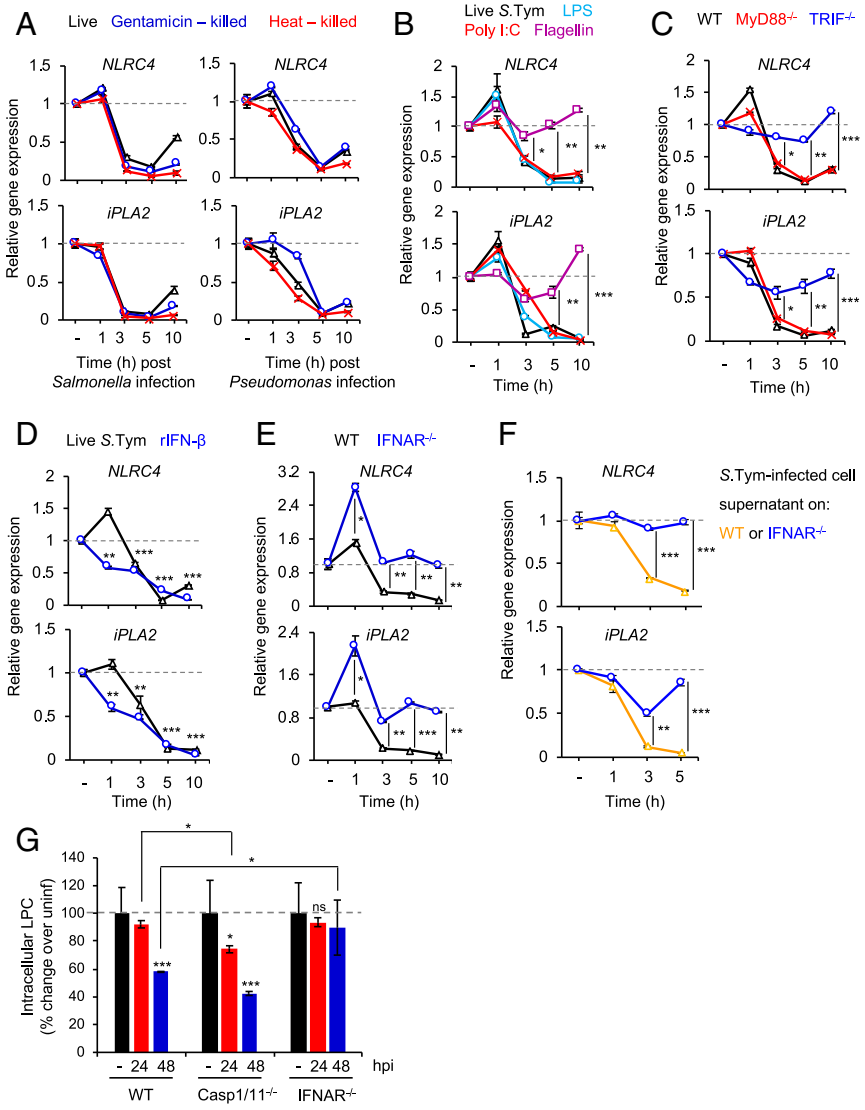

Fig. 5. Type 1 IFN signaling fosters a NLRC4-, iPLA2-, and LPC-low state in macrophages. (*A*) qPCR for NLRC4 and iPLA2 showing down-regulation of these genes in infected BMDMs over time. WT BMDMs were infected with 25 MOI of live, heat-killed (90 °C, 30 min) or gentamicin-killed (100 μg/mL, 30 min) *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*.

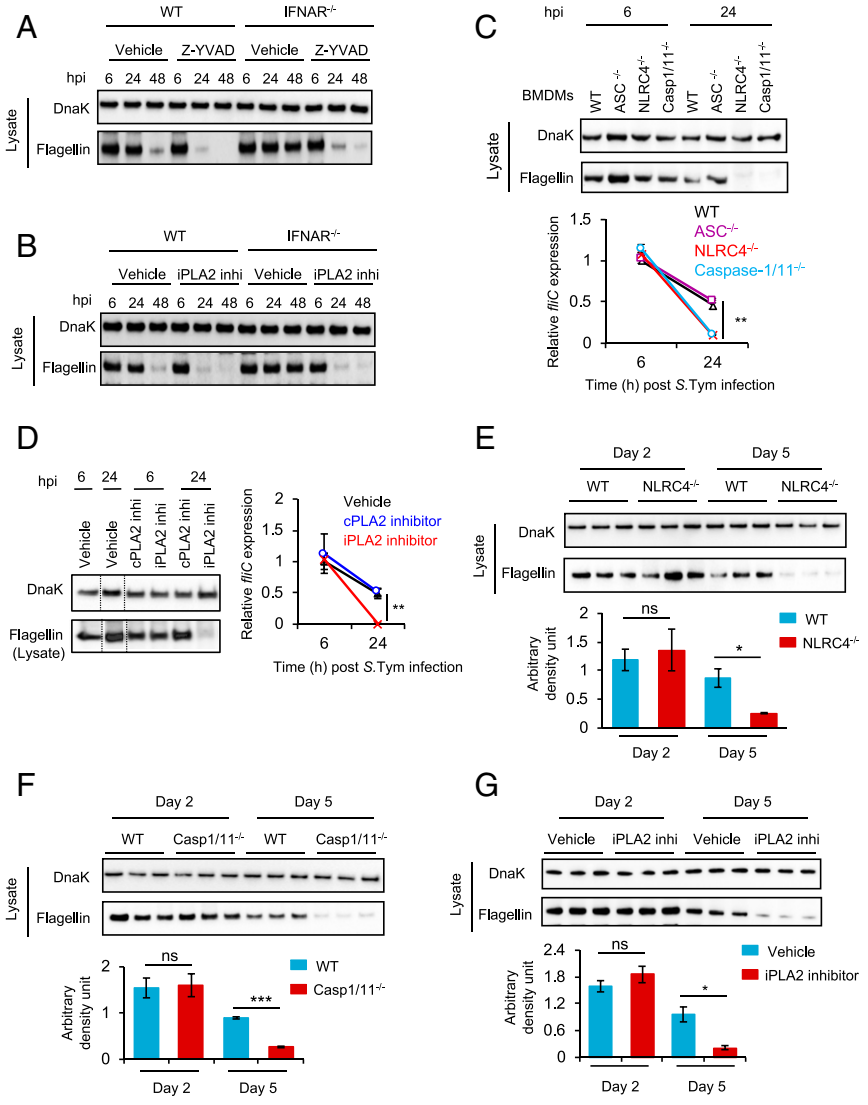

Fig. 6. NLRC4 inflammasome and iPLA2 activity controls expression of flagellin by intracellular *Salmonella*. (*A* and *B*) Immunoblot for flagellin and DnaK expressed by intracellular *Salmonella* obtained from BMDMs treated with caspase-1-specific (*A*) or iPLA2-specific (*B*) inhibitors. WT or IFNAR$^{-/-}$ 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*.

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