Cytoplasmic flagellin activates caspase-1 and secretion of interleukin 1β via Ipaf

Edward A Miao[1,2], Celia M Alpuche-Aranda[4], Monica Dors[1], April E Clark[1], Martin W Bader[2],

Macrophages respond to *Salmonella typhimurium* infection via Ipaf, a NACHT–leucine-rich repeat family member that activates caspase-1 and secretion of interleukin 1β. However, the specific microbial salmonella-derived agonist responsible for activating Ipaf is unknown. We show here that cytosolic bacterial flagellin activated caspase-1 through Ipaf but was independent of Toll-like receptor 5, a known flagellin sensor. Stimulation of the Ipaf pathway in macrophages after infection required a functional salmonella pathogenicity island 1 type III secretion system but not the flagellar type III secretion system; furthermore, Ipaf activation could be recapitulated by the introduction of purified flagellin directly into the cytoplasm. These observations raise the possibility that the salmonella pathogenicity island 1 type III secretion system cannot completely exclude 'promiscuous' secretion of flagellin and that the host capitalizes on this 'error' by activating a potent host-defense pathway.

The Toll-like receptors (TLRs) have emerged as quintessential pattern-recognition receptors in activating transcriptional responses to extracellular pathogen-associated molecular patterns or those contained in the vacuolar compartment[1]. The TLRs respond to a variety of extracellular pathogen-associated molecular patterns; for example, TLR4 responds to lipopolysaccharide (LPS), whereas TLR5 responds to bacterial flagellin[2,3]. TLRs have an extracellular leucine-rich repeat domain, which confers ligand specificity, and an intracellular TLR–interleukin 1 (IL-1) receptor signaling domain.

Another family of mammalian pattern-recognition receptors is now emerging, known as the NACHT–leucine-rich repeat receptors (NLRs; also called Nod, Nod-LRR or CATERPILLER), comprising the Nod, Nalp, Naip, Apaf and Ipaf proteins[4–7]. Like the TLRs, the NLRs also contain leucine-rich repeat domains that are linked to ligand recognition. A central nucleotide-binding domain mediates oligomerization after activation, resulting in the induced proximity of the active signaling caspase activation and recruitment domain (CARD) or pyrin domain (PYD). Nod1 and Nod2 activate transcriptional responses when activated by cytoplasmic bacterial peptidoglycan fragments[8–11]. Another NLR, Nalp3, has been shown to mediate responses to many stimuli, including gout-associated uric acid crystals, resiquimod, bacterial RNA, ATP and bacterial toxins[12–14]. The common link between these stimuli is unclear, although ATP and pore-forming toxins both partially permeabilize the cell membrane. Several NLRs, including Nalp1–Nalp3 and Ipaf, have been suggested as critical proteins required for the activation of IL-1β secretion[15,16].

IL-1β is a chief proinflammatory cytokine whose expression and secretion by macrophages is tightly regulated. That is demonstrated by the fact that TLR activation by extracellular pathogen-associated molecular patterns induces the transcription and translation of pro-IL-1β but not its secretion. Secretion requires a second signal that causes the activation of caspase-1, a cysteine protease that cleaves pro-IL-1β to mature IL-1β[17]. Activation of caspase-1 by *Salmonella typhimurium* requires signaling through the cytosolic mammalian protein Ipaf (also called CARD12 and CLAN)[18–21]. Exposure of macrophages to low numbers of *S. typhimurium* induces caspase-1–dependent IL-1β secretion, whereas a greater bacterial burden stimulates caspase-1–dependent cytotoxicity[18,22]. Those responses are dependent on a functional salmonella pathogenicity island 1 (SPI1) type III secretion system (TTSS) that delivers effector proteins to the eukaryotic cell cytosol. TTSSs are common bacterial virulence factors found in Gram-negative bacterial pathogens that transfer multiple protein effectors directly from the bacterial cytosol into the eukaryotic cell cytosol. These effectors produce a variety of effects on various aspects of host-cell physiology[23].

Macrophage sensing of SPI1 TTSS activity requires a competent bacterial secretory apparatus, including SipB, which is a component of the transmembrane pore inserted into the eukaryotic cell membrane. No known SPI1 TTSS translocated effector is required for salmonella-induced caspase-1 activation[24], indicating that caspase-1 responds to a conserved component of the secretion-translocation system itself or to an as-yet-unidentified translocated protein.

Flagella are assembled by a TTSS that is distinct from but evolutionarily related to the SPI1 TTSS. Flagellar gene expression and assembly are tightly regulated by three classes of promoters in a transcriptional cascade[25,26]. A class I regulatory system (FlhC–FlhD)

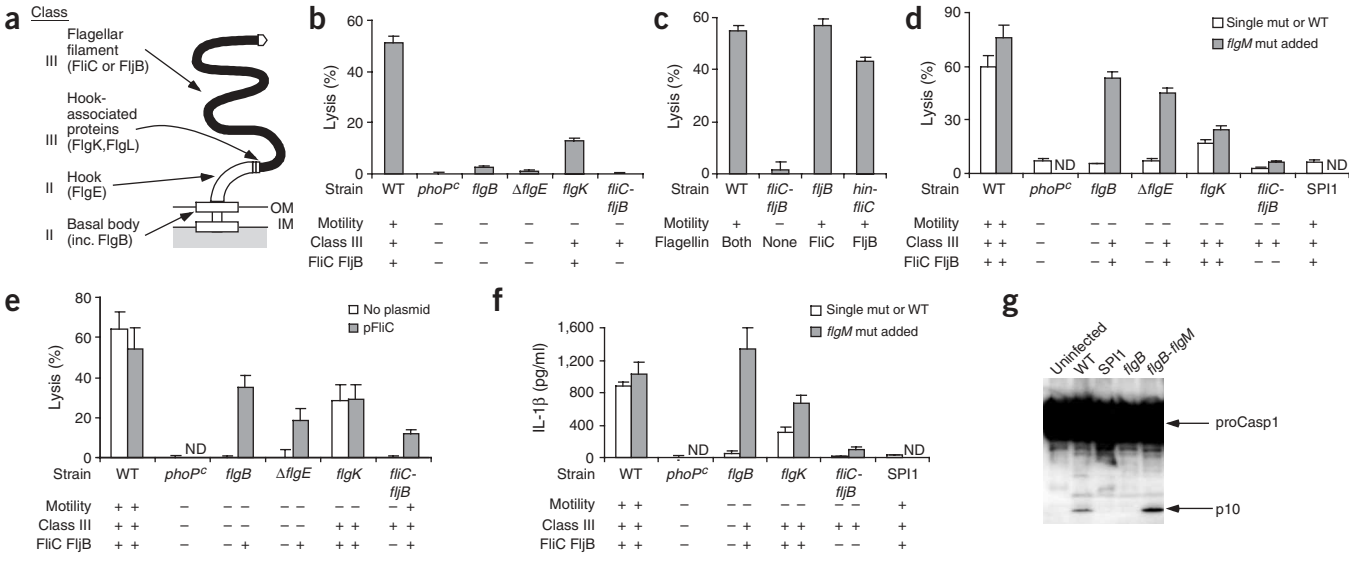

Figure 1 Salmonella expressing flagellin activate caspase-1 in BMMs independently of bacterial motility or intact hook–basal body structure. (**a**) Flagellar assembly, including the transcriptional class of each component and corresponding mutants. OM, outer membrane; IM, inner membrane. (**b**–**e**) Cytoxicity, determined by release of LDH from BMMs infected with wild-type (WT), SPI1 mutant (Δ*prgH–K*) or flagellar mutant (mut) *S. typhimurium* at an MOI of 40; cells were centrifuged to promote contact with bacteria. (**b**) Cytotoxic effect of various flagellin mutants on BMMs. (**c**) Cytotoxic effect on BMMs of flagellar mutants expressing one of the two flagellin phase variants (*fliC* or *fljB*). (**d**,**e**) Wild-type or various flagellar mutants with or without *flgM* mutation activating expression of flagellin and other class III genes (**d**) or plasmid-borne *fliC* (pFliC; **e**). (**f**) ELISA of IL-1β secretion after infection of LPS-stimulated BMMs with a lower MOI of 20, at which there is less cytotoxicity. Values are corrected for release of cytoplasmic pro-IL-1β due to cellular lysis. (**g**) Processing of caspase-1, assessed by immunoblot for the p10 processed fragment after infection of cells with bacteria (strain, above lanes) at an MOI of 20. proCasp1, pro-caspase-1. Blot is representative of three experiments. Error bars (**b**–**f**), mean ± s.d.; ND, not done.

activates class II genes that encode the secretory apparatus, basal body (including FlgB) and hook (FlgE). This secretion system then exports a transcriptional inhibitor (FlgM); once the bacterial cytosol is depleted of FlgM, class III genes are expressed. Class III genes encode additional components of the flagellum, including the hook-associated proteins (FlgK and FlgL), the flagellar filament proteins (FliC and FljB), the flagellar cap, the motor components that energize flagellar rotation and the chemotaxis apparatus. *S. typhimurium* alternately express two antigenic phase variants of the flagellin subunit encoded by *fliC* and *fljB*. We report here that bacterial flagellin present in the macrophage cytosol, most likely delivered by the SPI1 TTSS, stimulated Ipaf-dependent activation of caspase-1.

RESULTS

Caspase-1 activation requires flagellin but not motility

In a screen of *S. typhimurium* mutants, we found a strain defective in cytotoxicity to macrophages and motility. After inoculation in motility agar, spontaneous revertants of this mutant arose that had a motile and cytotoxic phenotype, suggesting that there was a point mutation in a flagellar gene. We therefore examined the potential effects of defined flagellar mutations on cytotoxicity after infection of macrophages to determine if cytotoxicity requires motility alone or, perhaps more notably, if it requires the expression of a specific flagellar gene (**Fig. 1**). Flagellar mutants unable to synthesize the basal body (*flgB*), hook (Δ*flgE*) or flagellin (*fliC-fljB*; **Fig. 1a**) were not cytotoxic to macrophages and did not activate IL-1β secretion, whereas bacteria with mutations in the hook-associated genes *flgK* and *flgL* retained their cytotoxicity and capacity to promote IL-1β secretion despite being nonmotile (**Fig. 1b**,**f**; structures formed by mutants, **Supplementary Fig. 1** online). The distinguishing difference between the noncytotoxic mutants and the cytotoxic mutants was that the latter expressed flagellin protein, whereas the former did not.

To determine whether the two flagellin phase variants (*fliC* and *fljB*) had similar cytotoxicity, we examined 'phase-locked' *S. typhimurium* strains. Phase variation is mediated by the Hin recombinase, which catalyzes the inversion of a small segment of the *S. typhimurium* chromosome carrying the promoter for the *fljBA* operon[25,26]. When *fljBA* is expressed, FljB monomers are expressed as the only flagellin filament protein, whereas FljA represses *fliC* expression. After inversion of the promoter region by Hin, *fljBA* expression is deactivated, resulting in expression of the other flagellin monomer protein, FliC. To activate *fljB* expression, we introduced a *hin* mutation into a *fliC* mutant strain to prevent phase variation ('phase-locked'). To ensure that FljB was expressed in the *hin-fljC* double-mutant strain, we then isolated a motile transductant. We also examined separate motile *fljB* mutants in parallel (introduction of a *hin* mutation into *fljB* mutants was unnecessary because *S. typhimurium* 14028s 'preferentially' expresses *fliC* and therefore *fljB* mutants are motile). Both the *hin-fliC* and *fljB* mutant strains retained cytotoxicity, indicating that either FliC or FljB expression was sufficient to induce caspase-1 activation

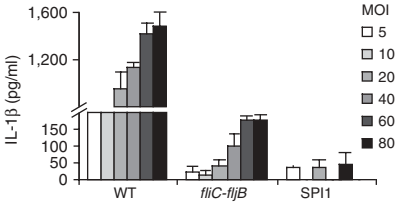

Figure 2 SPI1 TTSS–dependent, flagellin-independent IL-1β secretion occurs at a high MOI. LPS-stimulated wild-type BMMs were infected with wild-type, *fliC-fljB* or SPI1 (Δ*prgH–K*) mutant *S. typhimurium* at various MOI values and IL-1β secretion was determined by ELISA; values are corrected for cytoplasmic release due to cytotoxicity. Error bars, s.d.

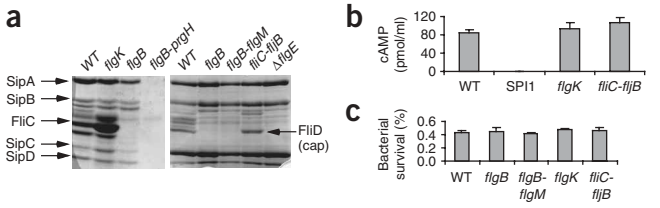

Figure 3 Flagellar mutants interact normally with cultured cells. (**a**) Coomassie-stained proteins secreted by wild-type bacteria, by flagellar mutants or by flagellar mutants carrying an SPI1 mutation (*prgH*), after TCA precipitation from overnight bacterial cultures. Arrows along left margin indicate location of SPI1 TTSS–secreted proteins and FliC. Data are two SDS-polyacrylamide gels with various mutants and are representative of at least three gels per strain. (**b**) SPI1 TTSS translocation activity. BMMs were infected with various *S. typhimurium* strains (at an MOI of 10) expressing the chimeric SspH1-CyaA protein; cAMP concentrations were determined after 1 h by enzyme immunoassay. (**c**) Survival, as assessed by colony-forming units from BMMs infected with wild-type *S. typhimurium* or various flagellar mutants at an MOI of 10. Error bars, s.e.m.

and IL-1β production from infected bone marrow–derived macrophages (BMMs; **Fig. 1c**). Furthermore, mutant strains overexpressing flagellin were more cytotoxic than were strains expressing less flagellin (**Supplementary Fig. 2** online). These experiments indicated that *S. typhimurium*–induced caspase-1 activation, IL-1β secretion and cytotoxicity require the expression of monomeric flagellin but not bacterial motility.

IL-1β secretion is independent of flagellar TTSS

We used two methods to express FliC and FljB in *S. typhimurium* strains already containing mutant hooks and basal bodies to further investigate whether export of flagellin monomers by the flagellar TTSS was required for cytotoxicity. We induced expression of endogenous *fliC* and *fljB* by introducing a *flgM* mutation that activated expression of all class III flagellar genes, including *fliC* and *fljB*. Mutation of *flgM* restored expression of FliC and FljB, as expected (**Supplementary Fig. 2**). In addition, we found that the *flgM* mutant also complemented hook and basal body mutants for macrophage cytotoxicity, activation of caspase-1 and stimulation of IL-1β secretion (**Fig. 1d**,**f**,**g**). Intrabacterial plasmid-borne expression of FliC alone also complemented hook and basal body mutants, indicating that monomeric FliC in the absence of other class III proteins was sufficient for the phenotype (**Fig. 1e**). Complementation of the hook and basal body mutants by expression of FliC in the bacterial cytoplasm was unexpected, as FliC cannot be exported by these mutants because the flagellar secretion system is not expressed in these strains. Furthermore, the addition of flagellin protein to the culture medium during infection of macrophages did not restore the cytotoxic phenotype of *fliC-fljB* mutants (data not shown), suggesting that in wild-type strains, a mechanism other than active secretion via the flagellar TTSS was needed to deliver flagellin to eukaryotic cells.

At higher multiplicity of infection (MOI), we found small amounts of IL-1β secretion after infection by salmonella strains deficient in production of both flagellin proteins (**Fig. 2**), a phenotype that nevertheless required a functional SPI1 TTSS, suggesting that in addition to a flagellin-dependent pathway of macrophage activation, *S. typhimurium* can deliver another mediator that activates caspase-1, albeit less efficiently than the flagellin-dependent pathway. In the remainder of our studies we used an MOI that did not stimulate IL-1β in the absence of flagellin expression.

Flagellar mutants have normal SPI1 TTSS activity

Because the virulence-associated SPI1 TTSS is required for activation of caspase-1 by *S. typhimurium*, we examined the activity of SPI1 TTSS in various flagellar mutant strains to verify that the phenotypes noted were not due to secondary effects. Published reports indicate that these or equivalent flagellar mutants are competent for invasion of epithelial cells and secretion of SipC into liquid media[27,28]. We thus verified that secretion of SPI1 TTSS in liquid culture was normal for our panel of flagellar mutants (**Fig. 3a**). We further determined if SPI1 TTSS was normally able to translocate effectors other than flagellin into BMMs by using a fusion 'reporter' protein consisting of CyaA (calmodulin-dependent adenylate cyclase toxin of *Bordetella pertussis*) fused to the known *S. typhimruium* SPI1 effector protein SspH1. We assayed translocation of this chimeric reporter molecule by the accumulation of cAMP in infected eukaryotic cells. We infected BMMs with SspH1-CyaA–expressing strains carrying various flagellar mutations and measured cAMP accumulation. We found that translocation of SspH1-CyaA from *flgK* and *fliC-fljB* mutants to the cytoplasm of BMMs was similar to that noted for wild-type salmonella (**Fig. 3b**). Furthermore, the entry and survival of flagellar mutant strains in BMMs was equal to that of the wild-type strain (**Fig. 3c**). Therefore, the SPI1 TTSS was not functionally defective in the flagellar mutant strains.

The PhoP-PhoQ intracellular sensor represses flagellin expression

Salmonella replicates in a membrane-bound vacuole in macrophages and senses the macrophage intracellular environment using the PhoP-PhoQ regulatory system, which activates several responses, including

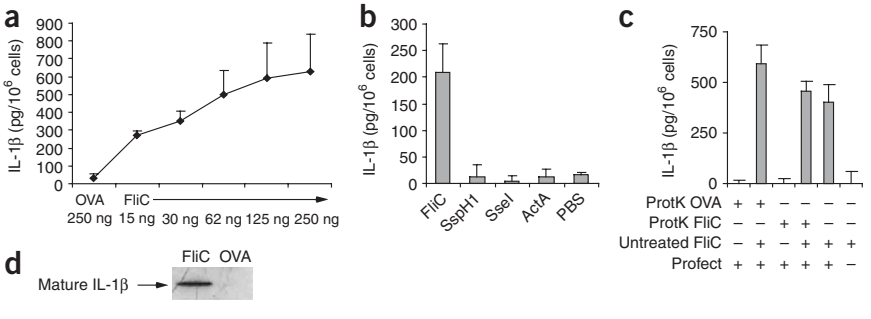

Figure 4 Cytoplasmic flagellin stimulates IL-1β secretion. (**a**) ELISA for IL-1β production from LPS-stimulated BMMs treated with ovalbumin (OVA) or various amounts of flagellin protein (FliC). (**b**) ELISA for IL-1β production from BMMs treated with PBS or 30 ng flagellin (FliC) or other bacterial virulence factors that access the cytosol of macrophages during normal infection: SspH1 (SPI1 TTSS effector), SseI (SPI2 TTSS effector) and ActA (listeria virulence factor). (**c**) ELISA for IL-1β production from BMMs treated with 125 ng ovalbumin or flagellin treated with proteinase K overnight (ProtK OVA and ProtK FliC), with or without 125 ng of flagellin that was not digested with proteinase K (Untreated FliC). Omission of the Profect transfection reagent (far right) is presented as a control. (**d**) Immunoblot for mature IL-1β secreted by BMMs transfected with 60 ng of flagellin (FliC) or ovalbumin. Cytotoxicity was negligible and equal for all samples (<5%). Error bars (**a**–**c**), s.d.

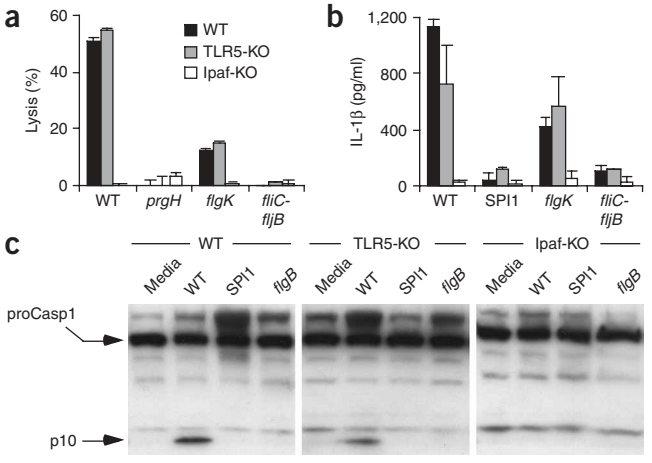

Figure 5 BMMs respond to flagellin by secreting IL-1β independently of TLR5. LPS-stimulated BMMs derived from wild-type, TLR5-deficient (TLR5-KO) or Ipaf-deficient (Ipaf-KO) mice were infected with wild-type, SPI1 mutant (Δ*prgH–K*) or flagellar mutant *S. typhimurium*. (**a**) Cytoxicity, assessed by LDH release from wild-type, TLR5-deficient or Ipaf-deficient BMMs infected with bacteria (strains, horizontal axis) at an MOI of 40. (**b**) ELISA for IL-1β secretion from BMMs after infection at an MOI of 20. Values are corrected for release of cytoplasmic pro-IL-1β. Error bars, s.d. (**c**) Immunoblot for caspase-1 p10 of BMMs infected with *S. typhimurium* at an MOI of 20. Blots are representative of three experiments.

LPS modifications that decrease recognition of TLR4 and its coreceptor MD2 (ref. 29). Because flagellin can activate robust IL-1β secretion, we hypothesized that during infection *S. typhimurium* would repress flagellin expression when in the macrophage intracellular environment to prevent activation of IL-1β secretion. We found that activation of the PhoP-PhoQ system strongly repressed *fliC* expression (by 99%), exerted less regulatory control over class II genes (*flgC*) and did not regulate class I gene expression (*flhC*; **Supplementary Fig. 3** online). Such transcriptional regulation is consistent with the reported lack of flagellar secretion in strains carrying a mutation that constitutively activates the PhoP-PhoQ regulatory system[30] and with the lack of cytotoxicity in these mutants (**Fig. 1**). It is noteworthy that PhoP-PhoQ repressed both SPI1 and flagellar gene expression after phagocytosis, which would result in a decrease in immunostimulatory activity.

Cytoplasmic flagellin activates caspase-1 independently of TLR5

Because flagellin present outside macrophages does not simulate IL-1β and caspase-1 activation and because we found that the flagellar TTSS was not required for IL-1β secretion (although the virulence factor SPI1 TTSS was), we hypothesized that macrophages were responding to flagellin that entered the cytoplasm, perhaps via the SPI1 TTSS (discussed below). To formally demonstrate that macrophages secrete IL-1β in response to cytoplasmic flagellin, we introduced purified protein into the cytosol of BMMs (**Fig. 4**). Transfected flagellin stimulated the secretion of mature IL-1β, whereas a control (ovalbumin) or flagellin added without the transfection reagent did not (**Fig. 4a**,**c**,**d**). Both the flagellin and ovalbumin preparations were free of contamination with LPS or other TLR agonists. Other bacterial virulence factors from *S. typhimurium* (SspH1 and SseI) or *Listeria monocytogenes* (ActA) that normally access the cytosol during infection did not activate IL-1β secretion when transfected into BMMs, nor did they inhibit flagellin mediated activation (**Fig. 4b** and data not shown). Proteinase K eliminated flagellin-induced IL-1β secretory activity (**Fig. 4c**), further supporting the conclusion that flagellin alone was responsible for activating IL-1β secretion.

We sought to define the mammalian receptor responsible for recognition of cytoplasmic flagellin. TLR5 is a transmembrane receptor for extracellular flagellin that signals through the adaptor protein MyD88 (ref. 3). However, mouse macrophages do not express TLR5 and are not responsive to extracellular flagellin (unlike human monocytes)[31,32]. Nevertheless, to investigate the function of TLR5 signaling in *S. typhimurium*–induced macrophage cytotoxicity and IL-1β secretion, we infected BMMs derived from TLR5-deficient mice. Even in the absence of TLR5, we noted flagellin-dependent cytotoxicity, IL-1β secretion and caspase-1 processing (**Fig. 5**); we obtained a similar result with MyD88-deficient BMMs (data not shown). These results demonstrated that TLR5 and other TLRs signaling through MyD88 are not required for the flagellin-dependent response.

Ipaf responds to cytoplasmic flagellin

Ipaf activates caspase-1 in response to *S. typhimurium* infection but is not required for caspase-1 activation in response to ATP or infection with *Francisella tularensis*[13,33]. BMMs derived from Ipaf-deficient mice are resistant to *S. typhimurium*–induced cytotoxicity and IL-1β secretion[18] (**Fig. 5**), an effect not explained by decreased uptake of bacteria (**Supplementary Fig. 4** online). Thus, we hypothesized that Ipaf was required for the response to cytosolic bacterial flagellin. The J774A.1 and RAW264.7 macrophage cell lines are much more resistant than BMMs to *S. typhimurium*–induced IL-1β secretion; however, when Ipaf was overexpressed in these cells, they became sensitized to salmonella cytotoxicity and IL-1β secretion, whereas control cells expressing green fluorescent protein were not (**Fig. 6** and **Supplementary Fig. 4**). We also found that although BMMs from wild-type or TLR5-deficient mice secreted IL-1β in response to transfected flagellin protein, BMMs derived from Ipaf-deficient mice did not respond to cytoplasmic flagellin (**Fig. 7a**). In agreement with the lack of IL-1β secretion, Ipaf-deficient BMMs did not activate caspase-1 processing in response flagellin transfection (**Fig. 7b**). We confirmed the specificity of Ipaf by activating IL-1β secretion with resiquimod (**Fig. 7c**), a known Nalp3 agonist[12].

Finally, the NLR adaptor protein ASC is essential for Nalp3-mediated caspase-1 activation in response to multiple agonists[12–14]. ASC-deficient macrophages are also partially deficient in *S. typhimurium*–induced macrophage cell death[18]. We therefore assessed IL-1β secretion in response to transfection of purified flagellin in ASC-deficient BMMs and found a partial defect in IL-1β secretion in response to cytoplasmic flagellin (**Fig. 7d**). Thus, ASC modulates the response to cytoplasmic flagellin, but is not essential.

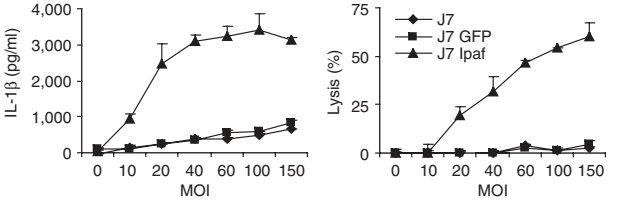

Figure 6 Ipaf sensitizes J774A.1 cells to *S. typhimurium*. J774A.1 macrophages transfected with Ipaf or green fluorescent protein (GFP) were stimulated for 3 h with 300 ng/ml of PAM_3CSK_4 and then were infected with *S. typhimurium* (MOI, horizontal axes) and were evaluated for IL-1β secretion (left) or lysis (assessed as LDH release; right). Values are normalized to account for unprocessed IL-1β released because of cellular lysis. Error bars, s.d.

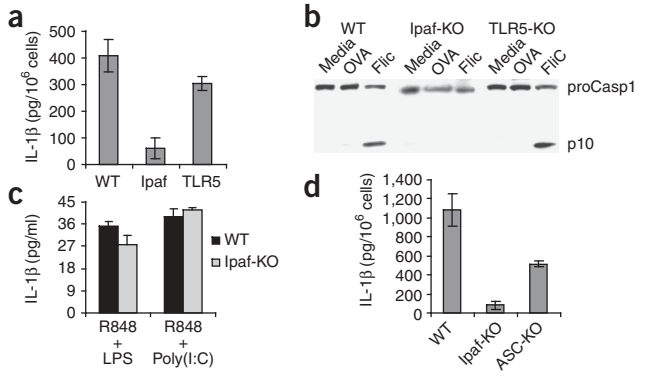

Figure 7 Ipaf is required for the response to cytoplasmic flagellin. (**a**,**b**) BMMs from wild-type, Ipaf-deficient (Ipaf-KO) or TLR5-deficient (TLR5-KO) mice were stimulated with LPS for 2 h before transfection with 60 ng purified flagellin. (**a**) ELISA for IL-1β secretion. $P > 0.05$, wild-type versus TLR5-deficient BMMs; $P < 0.05$, Ipaf-deficient BMMs versus wild-type and TLR5-deficient BMMs. (**b**) Immunoblot of processed caspase-1 after transfection for 2 h with purified flagellin or ovalbumin. Blots are representative of three experiments. (**c**) ELISA of IL-1β secretion by wild-type or Ipaf-deficient BMMs stimulated for 24 h with resiquimod (R848; 5 μg/ml) plus LPS (50 ng/ml) or poly(I)·poly(C) (Poly(I:C); 5 μg/ml). (**d**) ELISA of IL-1β secretion from BMMs from wild-type, Ipaf-deficient or ASC-deficient (ASC-KO) mice stimulated with 10 ng/ml of LPS for 2 h before transfection with 30 ng purified flagellin. $P < 0.05$, wild-type versus ASC-deficient. Cytotoxicity was negligible and equal for all samples (<5%).

DISCUSSION

The data presented here have identified Ipaf as an essential sensor for cytoplasmic flagellin. Mammalian cells are thus able to sense extracellular flagellin through TLR5 and intracellular flagellin through Ipaf. These two sensory pathways result in different responses. TLR5 activates transcription factor NF-κB, resulting in the secretion of many cytokines and other transcriptional responses, including the expression of pro-IL-1β but not secretion of mature IL-1β. Ipaf, in contrast, initiates IL-1β processing and stimulates the secretion of mature IL-1β. By using two sensory pathways for bacterial flagellin, the innate immune system may be able to modulate the intensity or quality of its response according to the virulence characteristics of the pathogen. The presence of less-virulent flagellated commensal bacteria in tissues would warrant a less-vigorous response, with responses to flagellin mediated by TLR5 but not Ipaf. In contrast, for pathogens such as salmonella that express many virulence factors and can translocate effector proteins into the eukaryotic cell cytosol, a more-robust response may be required to control the infection. The presence of flagellin in the host cell cytosol is therefore a marker for virulence detected by the innate immune system through Ipaf, resulting in the addition of IL-1β secretion to TLR-mediated responses. Another difference between TLR5 and Ipaf activation is that hyperstimulation of Ipaf results in caspase-1-dependent cell death, whose relevance *in vivo* remains to be determined. However, caspase-1-dependent cell death might be beneficial to the host because it eliminates immune cells that have been exposed to TTSS effectors or whose cytosol has been compromised by the presence of flagellated bacteria.

ASC is a small protein that contains a PYD and a CARD, which serves as an adaptor for some NLRs. For example, the PYDs of Nalp1, Nalp2 and Nalp3 interact with the PYD of ASC, which subsequently recruits caspase-1 through CARD-CARD homotypic interactions[15,16]. Unlike the Nalp proteins, Ipaf contains a CARD signaling domain that directly interacts with the CARD of caspase-1 (refs. 19,20). However, the CARD of Ipaf also interacts with the CARD of ASC[20,34,35]. Thus, the function of ASC in Ipaf-mediated caspase-1 activation and IL-1β secretion remains unclear. We found that ASC-deficient BMMs were partially defective in their response to cytoplasmic flagellin, consistent with published studies demonstrating a partial requirement for ASC in *S. typhimurium*–induced caspase-1-dependent cell death[18]. As there is evidence of trimeric interactions between members of the structurally related death domain family[36], ASC may stabilize the interaction between the CARDs of Ipaf and caspase-1 by forming a trimeric CARD complex. Alternatively, ASC-deficient BMMs may have basal dysregulation in the caspase-1 activation pathway. In either case, ASC is not completely required for Ipaf-dependent activation of caspase-1 in response to cytoplasmic flagellin.

So far very little evidence exists for direct binding of agonists to TLRs or NLRs, and several TLR coreceptors have been identified. For example, TLR4 cooperates with CD14, LBP and MD2 to recognize LPS[1], although the mechanism of the interactions between these proteins and LPS remains unclear. The fact that TLRs and NLRs both contain leucine-rich repeat domains suggests that they participate in direct recognition, possibly in the context of multicomponent complexes. Thus, activation of Ipaf by flagellin may be direct and it may involve Ipaf in complex with other proteins or there may be an 'upstream' receptor for cytosolic flagellin that activates Ipaf.

Activation of caspase-1 by *S. typhimurium* was previously attributed to SipB. In shigella, the SipB homolog IpaB is also believed to be responsible for caspase-1 activation. IpaB and SipB are integral components of their respective TTSS transmembrane pore complex that is inserted into the eukaryotic cell membrane. Both interact with caspase-1 (refs. 37,38), which has been suggested as the mechanism by which caspase-1 is activated by salmonella and shigella. Microinjection of purified SipB protein causes cell death[37], although the mechanism of that is probably caspase-1-independent autophagy[24]. The interaction between SipB and caspase-1 has not been shown to result in activation of caspase-1 in the absence of flagellin-expressing bacteria. *S. typhimurium sipB* mutants are unable to translocate any proteins via the SPI1 TTSS, which makes it impossible to definitively attribute caspase-1 activation to SipB protein alone by the use of *sipB* mutants.

We have now demonstrated that flagellin recognition is responsible for most SPI1-mediated Ipaf-dependent caspase-1 activation. We also found that less IL-1β secretion noted at higher bacterial MOI values was dependent on SPI1 but was independent of flagellin. This flagellin-independent IL-1β secretion mechanism may be mediated by SipB, either by direct activation of caspase-1 or by indirect sensing through an unidentified NLR family member. Because SipB forms pores in the membrane of infected macrophages, SPI1 TTSS activity may result in the disruption of ionic gradients across the macrophage plasma membrane, which would be predicted to activate Nalp3 by a mechanism similar to activation of the P2X7 ATP-gated ion channel (although such a hypothesis does not account for direct interaction between SipB and caspase-1). In addition, calcium influx resulting from SPI1 TTSS plasma membrane permeabilization triggers exocytosis of lysosomes[39], a process that has been linked to ATP-activated IL-1β secretion[40].

We have yet to elucidate the mechanism by which salmonella flagellin, in cooperation with SPI1 TTSS activity, accesses the cytosol. It is possible that the activity of SPI1 transiently permeabilizes the vacuolar membrane, permitting free flagellin in the vacuole to access the host cell cytosol. However, the flagellar secretion apparatus is not required for Ipaf activation. Furthermore, *S. typhimurium* with more cytosolic flagellin have more caspase-1 activation potential than do strains that export large amounts of flagellin. Those observations

suggest a more direct mechanism of delivery. We speculate that a small amount of flagellin is translocated through the SPI1 TTSS 'needle complex' into the host cell cytoplasm. The converse phenomenon, secretion of SPI1 proteins by the flagellar secretion system, has been reported[41]. This is probably an evolutionary 'accident' arising from the flexibility of the TTSS secretion signal[42], making it difficult to completely exclude the flagellar substrates from the virulence-associated TTSS. The host seems to have taken advantage of that error, recognizing the highly conserved flagellin protein rather than the more weakly conserved components of the virulence-associated TTSS. Our findings suggest that the innate immune system responds vigorously to bacterial flagellin; TLR5 senses extracellular flagellin, and a separate Ipaf-dependent activation pathway responds to flagellin in the cytoplasm. Delivery of flagellin to the cytosol by virulence-associated TTSS may not be the only mechanism whereby flagellin accesses the cytosol. It is plausible that Ipaf mediates responses to organisms that deliver flagellin to the cytosol by alternative pathways, including those that escape from the phagocytic vacuole such as *L. monocytogenes* and *Shigella* spp.

METHODS

Strain construction and bacterial growth conditions. Bacterial strains were constructed by P22HT *int* transduction. For Δ*flgE1204* transduction, first *pyrC691*::Tn*10* was transduced into 14028s, followed by transduction with Δ*flgE1204*, plating on tetracycline-sensitivity media and screening for non-motile clones. Motility phenotype and flagellin subunit expression of all transductants were verified by inoculation in motility agar and immunoblot (data not shown). We have compiled a list of all flagellar mutants used (**Supplementary Table 1** online). Salmonella strains were grown in Luria-Bertani medium overnight, were diluted 1:40 (volume/volume) and were grown for 3 h to induce SPI-1 TTSS expression for infection experiments.

Tissue culture and mice. BMMs were prepared from the femurs of BALB/c mice (**Supplementary Fig. 1**), C57BL/6 mice, TLR5-deficient (null) mice, Ipaf-deficient (null) mice[8] and ASC-deficient (null) mice[18] by culture with L-cell supernatant or by the addition of recombinant human macrophage colony-stimulating factor (50 ng/ml). BALB/c mice were purchased from Jackson Laboratories; C57BL/6 mice, from Charles River Laboratories. Mice were housed in a specific pathogen–free environment with approval and supervision of the Institutional Animal Care and Use Committee at the Institute for Systems Biology (Seattle, Washington).

Bacterial infection. Colony-forming units were determined for BMMs infected with wild-type *S. typhimurium* or various flagellar mutants at an MOI of 10, where cytotoxicity was not found. After 1 h, BMMs were washed and were treated for 30 min with 100 μg/ml of gentamicin to kill extracellular bacteria. BMMs were washed and lysed with 0.5% deoxycholate, and colony-forming units were determined by dilutional plating and were normalized as a percent of the infecting bacteria present.

Retroviral transfection. Phoenix Ampho cells (American Type Culture Collection) were transfected with pMXsIP mIpaf-PC or PC-GFP (where PC is the protein C epitope tag and GFP is green fluorescent protein) using Lipofectamine 2000 (Invitrogen). J774A.1 or RAW264.7 cells were transfected with the resulting retrovirus and a transfected population was selected with puromycin and was confirmed by flow cytometry and immunoblot. RAW264.7 clones expressing Ipaf were isolated by dilutional plating and the Ipaf expressed was confirmed as being free of mutations.

Cytotoxicity assay and IL-1β enzyme-linked immunosorbent assay (ELISA). BMMs, J774A.1 cells or RAW264.7 cells were plated and then were infected the next day. For induction of pro-IL-1β expression, BMMs were stimulated for 2 h with 10 ng/ml of ultrapure *Salmonella minnesota* LPS (List Biological Laboratories) that does not activate pro-IL-1β processing and secretion. Unlike BMMs, J774A.1 and RAW264.7 cells were found to respond more vigorously to synthetic tripalmitoyl cysteinyl lipopeptide (Pam_3CSK_4; EMC Microcollections), which was used to induce pro-IL-1β for 2 h. SPI1-induced bacteria (MOI, 40, unless indicated otherwise) were diluted in 100 μl media and were added to the BMMs. When flagellar mutants were included in the assay, plates were centrifuged for 5 min at 1,500 r.p.m. in a Beckman GS-6R swinging-bucket centrifuge. Infections were allowed to proceed for 30–60 min at 37 °C in an atmosphere of 5% CO_2. Lactate dehydrogenase (LDH) activity in the supernatant was determined with the CytoTox 96 assay (Promega), and IL-1β secretion was determined by ELISA (R&D Systems). Similar cytotoxicity results were obtained when propidium iodine staining and flow cytometry were used (data not shown). Each sample was done in triplicate and results are representative of at least three experiments. For controlling for release of pro-IL-1β from dead cells, a parallel well was lysed with 0.9% Triton-X100 and cytoplasmic pro-IL-1β was collected and analyzed by ELISA for mature IL-1β, which is less sensitive for pro-IL-1β. The IL-1β values shown were normalized for the release of pro-IL-1β from lysed macrophages (mature IL-1β = total IL-1β signal – pro-IL-1β lysis × percent release of LDH).

Immunoblot. For detection of processed caspase-1, BMMs were seeded into 24-well plates and were infected for 1 h with *S. typhimurium* at an MOI of 20 or purified protein was transfected for 2 h using Profect. Infections and protein transfections were done in serum-free media. Cells were lysed and nuclei were cleared by centrifugation. Because processed caspase-1 is exported as well as being cell associated, cytoplasmic lysates were combined with supernatants from the infections and protein was precipitated with 10% trichloroacetic acid (TCA) and were detected by immunoblot with antisera to caspase-1 p10 (Santa Cruz Biotechnology). IL-1β was precipitated from BMM supernatants with TCA and was detected by immunoblot with goat antibody to mouse IL-1β (R&D systems).

Preparation of bacterial secreted proteins. Bacterial strains were grown overnight in Luria-Bertani medium, cells were pelleted by ultracentrifugation and secreted proteins were precipitated with TCA as described[30]. Proteins were separated by SDS-PAGE and were stained with Coomassie brilliant blue. The *flgK* mutants secrete copious FliC into the supernatant. The *fliC-fljB* mutants hypersecrete the flagellar cap protein FliD, which was verified to not react with antisera specific for FliC (**Supplementary Fig. 2**).

CyaA reporter assays. These assays were done as described[43,44]. BMMs were infected for 1 h with bacteria expressing SspH1-CyaA at an MOI of 10 before lysis in 100 mM HCl. Concentrations of cAMP were determined with the Direct cyclic AMP Colorimetric (EIA) Kit (Assay Designs).

Protein transfection. Flagellin was purified from *S. typhimurium* culture as described[3]. Flagellin and ovalbumin were further purified by filtration through a filter with a molecular weight cutoff of 100 kilodaltons (Chemicon) and passage through a Detoxi-Gel Endotoxin Removing column (Pierce). LPS contamination was undetectable by the limulus assay (Cambrex). Contamination by pathogen-associated molecular patterns was further assayed by using the purified protein to stimulate BMMs overnight at doses from 20 μg/ml to 500 μg/ml; no secretion of tumor necrosis factor, IL-6 or IL-12 was detectable. These preparation were used to stimulate RAW264.7 cells stably expressing ELAM-luciferase; no NF-κB activation was detectable. The preparations contained no detectable MDP or other Nod2 stimuli, as proteolyzed flagellin did not activate an NF-κB luciferase reporter in Nod2-expressing HEK293T cells. Glutathione *S*-transferase, glutathione *S*-transferase–SspH1, six-histidine–tagged SseI and histidine-tagged ActA were purified as described[45]. BMMs were seeded in 24- or 96-well plates and were stimulated for 2 h with LPS (10 ng/ml) before being washed two times in serum-free media, and cells were transfected for 2 h with protein using Profect P1 (Targeting Systems) before determination of IL-1β secretion. For **Figure 7b** only, protein transfection was enhanced by centrifugation at 1,500 r.p.m. for 5 min in a Beckman GS-6KR centrifuge to promote contact with cells. IL-1β is expressed as 'picograms per million BMMs' to correct for different volumes of media required for seeding in 96-well versus 24-well plates; a value of 600 pg/10^6 cells correlates with 150–400 pg/ml. For protease digestion, proteinase K (Sigma) or PBS was added at 37 °C overnight in DMEM, after which 1 mM phenylmethyl sulfonyl fluoride was added to stop the reaction. LDH release was similar for each treatment. Student's *t*-test was

used for statistical analysis; results were considered statistically significant at a P value of less than 0.05.

Note: Supplementary information is available on the Nature Immunology website.

ACKNOWLEDGMENTS

We thank V. Dixit and Genentech for Ipaf- and ASC-deficient mice; S. Akira for TLR5- and MyD88-deficient mice; G. Chilcott, H. Bonnifield, O. Nanassy, J. Karlinsey and K. Hughes for flagellar mutant strains and advice regarding flagellar biology; C. Carlson, M. Ohl and K. Smith for contributions and discussions; H. Bonnifield, C. Carlson and members of the Aderem lab for critical review of this manuscript; and E. Andersen-Nissen for providing flagellin and ovalbumin protein.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Published online at http://www.nature.com/natureimmunology/
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/

1. Akira, S. & Takeda, K. Toll-like receptor signalling. *Nat. Rev. Immunol.* **4**, 499–511 (2004).
2. Poltorak, A. *et al.* Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* **282**, 2085–2088 (1998).
3. Hayashi, F. *et al.* The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* **410**, 1099–1103 (2001).
4. Martinon, F. & Tschopp, J. NLRs join TLRs as innate sensors of pathogens. *Trends Immunol.* **26**, 447–454 (2005).
5. Inohara, N., Chamaillard, M., McDonald, C. & Nunez, G. NOD-LRR proteins: role in host-microbial interactions and inflammatory disease. *Annu. Rev. Biochem.* **74**, 355–383 (2005).
6. Harton, J.A., Linhoff, M.W., Zhang, J. & Ting, J.P. Cutting edge: CATERPILLER: a large family of mammalian genes containing CARD, pyrin, nucleotide-binding, and leucine-rich repeat domains. *J. Immunol.* **169**, 4088–4093 (2002).
7. Kufer, T.A., Fritz, J.H. & Philpott, D.J. NACHT-LRR proteins (NLRs) in bacterial infection and immunity. *Trends Microbiol.* **13**, 381–388 (2005).
8. Chamaillard, M. *et al.* An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. *Nat. Immunol.* **4**, 702–707 (2003).
9. Girardin, S.E. *et al.* Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. *Science* **300**, 1584–1587 (2003).
10. Girardin, S.E. *et al.* Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *J. Biol. Chem.* **278**, 8869–8872 (2003).
11. Inohara, N. *et al.* Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease. *J. Biol. Chem.* **278**, 5509–5512 (2003).
12. Kanneganti, T.D. *et al.* Bacterial RNA and small antiviral compounds activate caspase-1 through cryopyrin/Nalp3. *Nature* **440**, 233–236 (2006).
13. Mariathasan, S. *et al.* Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature* **440**, 228–232 (2006).
14. Martinon, F., Petrilli, V., Mayor, A., Tardivel, A. & Tschopp, J. Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature* **440**, 237–241 (2006).
15. Martinon, F., Burns, K. & Tschopp, J. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol. Cell* **10**, 417–426 (2002).
16. Agostini, L. *et al.* NALP3 forms an IL-1β-processing inflammasome with increased activity in Muckle-Wells autoinflammatory disorder. *Immunity* **20**, 319–325 (2004).
17. Martinon, F. & Tschopp, J. Inflammatory caspases: linking an intracellular innate immune system to autoinflammatory diseases. *Cell* **117**, 561–574 (2004).
18. Mariathasan, S. *et al.* Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. *Nature* **430**, 213–218 (2004).
19. Poyet, J.L. *et al.* Identification of Ipaf, a human caspase-1-activating protein related to Apaf-1. *J. Biol. Chem.* **276**, 28309–28313 (2001).
20. Geddes, B.J. *et al.* Human CARD12 is a novel CED4/Apaf-1 family member that induces apoptosis. *Biochem. Biophys. Res. Commun.* **284**, 77–82 (2001).
21. Damiano, J.S., Stehlik, C., Pio, F., Godzik, A. & Reed, J.C. CLAN, a novel human CED-4-like gene. *Genomics* **75**, 77–83 (2001).
22. Chen, L.M., Kaniga, K. & Galan, J.E. Salmonella spp. are cytotoxic for cultured macrophages. *Mol. Microbiol.* **21**, 1101–1115 (1996).
23. Galan, J.E. Salmonella interactions with host cells: type III secretion at work. *Annu. Rev. Cell Dev. Biol.* **17**, 53–86 (2001).
24. Hernandez, L.D., Pypaert, M., Flavell, R.A. & Galan, J.E. A Salmonella protein causes macrophage cell death by inducing autophagy. *J. Cell Biol.* **163**, 1123–1131 (2003).
25. Chilcott, G.S. & Hughes, K.T. Coupling of flagellar gene expression to flagellar assembly in *Salmonella enterica* serovar typhimurium and *Escherichia coli*. *Microbiol. Mol. Biol. Rev.* **64**, 694–708 (2000).
26. Aldridge, P. & Hughes, K.T. Regulation of flagellar assembly. *Curr. Opin. Microbiol.* **5**, 160–165 (2002).
27. Eichelberg, K. & Galan, J.E. The flagellar sigma factor FliA (σ_{28}) regulates the expression of Salmonella genes associated with the centisome 63 type III secretion system. *Infect. Immun.* **68**, 2735–2743 (2000).
28. van Asten, F.J., Hendriks, H.G., Koninkx, J.F. & van Dijk, J.E. Flagella-mediated bacterial motility accelerates but is not required for Salmonella serotype Enteritidis invasion of differentiated Caco-2 cells. *Int. J. Med. Microbiol.* **294**, 395–399 (2004).
29. Guo, L. *et al.* Regulation of lipid A modifications by *Salmonella typhimurium* virulence genes phoP-phoQ. *Science* **276**, 250–253 (1997).
30. Pegues, D.A., Hantman, M.J., Behlau, I. & Miller, S.I. PhoP/PhoQ transcriptional repression of *Salmonella typhimurium* invasion genes: evidence for a role in protein secretion. *Mol. Microbiol.* **17**, 169–181 (1995).
31. Means, T.K., Hayashi, F., Smith, K.D., Aderem, A. & Luster, A.D. The Toll-like receptor 5 stimulus bacterial flagellin induces maturation and chemokine production in human dendritic cells. *J. Immunol.* **170**, 5165–5175 (2003).
32. Applequist, S.E., Wallin, R.P. & Ljunggren, H.G. Variable expression of Toll-like receptor in murine innate and adaptive immune cell lines. *Int. Immunol.* **14**, 1065–1074 (2002).
33. Mariathasan, S., Weiss, D.S., Dixit, V.M. & Monack, D.M. Innate immunity against *Francisella tularensis* is dependent on the ASC/caspase-1 axis. *J. Exp. Med.* **202**, 1043–1049 (2005).
34. Srinivasula, S.M. *et al.* The PYRIN-CARD protein ASC is an activating adaptor for caspase-1. *J. Biol. Chem.* **277**, 21119–21122 (2002).
35. Wang, L. *et al.* PYPAF7, a novel PYRIN-containing Apaf1-like protein that regulates activation of NF-κB and caspase-1-dependent cytokine processing. *J. Biol. Chem.* **277**, 29874–29880 (2002).
36. Weber, C.H. & Vincenz, C. The death domain superfamily: a tale of two interfaces? *Trends Biochem. Sci.* **26**, 475–481 (2001).
37. Hersh, D. *et al.* The *Salmonella* invasin SipB induces macrophage apoptosis by binding to caspase-1. *Proc. Natl. Acad. Sci. USA* **96**, 2396–2401 (1999).
38. Hilbi, H. *et al.* Shigella-induced apoptosis is dependent on caspase-1 which binds to IpaB. *J. Biol. Chem.* **273**, 32895–32900 (1998).
39. Roy, D. *et al.* A process for controlling intracellular bacterial infections induced by membrane injury. *Science* **304**, 1515–1518 (2004).
40. Andrei, C. *et al.* Phospholipases C and A2 control lysosome-mediated IL-1 β secretion: Implications for inflammatory processes. *Proc. Natl. Acad. Sci. USA* **101**, 9745–9750 (2004).
41. Lee, S.H. & Galan, J.E. Salmonella type III secretion-associated chaperones confer secretion-pathway specificity. *Mol. Microbiol.* **51**, 483–495 (2004).
42. Lloyd, S.A., Norman, M., Rosqvist, R. & Wolf-Watz, H. Yersinia YopE is targeted for type III secretion by N-terminal, not mRNA, signals. *Mol. Microbiol.* **39**, 520–531 (2001).
43. Miao, E.A. & Miller, S.I. A conserved amino acid sequence directing intracellular type III secretion by *Salmonella typhimurium*. *Proc. Natl. Acad. Sci. USA* **97**, 7539–7544 (2000).
44. Miao, E.A. *et al.* Salmonella typhimurium leucine-rich repeat proteins are targeted to the SPI1 and SPI2 type III secretion systems. *Mol. Microbiol.* **34**, 850–864 (1999).
45. Miao, E.A. *et al.* Salmonella effectors translocated across the vacuolar membrane interact with the actin cytoskeleton. *Mol. Microbiol.* **48**, 401–415 (2003).