

Innate immune recognition of flagellin limits systemic persistence of *Brucella*

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Summary

***Brucella* are facultative intracellular bacteria that cause chronic infections by limiting innate immune recognition. It is currently unknown whether *Brucella* FliC flagellin, the monomeric subunit of flagellar filament, is sensed by the host during infection. Here, we used two mutants of *Brucella melitensis*, either lacking or overexpressing flagellin, to show that FliC hinders bacterial replication *in vivo*. The use of cells and mice genetically deficient for different components of inflammasomes suggested that FliC was a target of the cytosolic innate immune receptor NLRC4 *in vivo* but not in macrophages *in vitro* where the response to FliC was nevertheless dependent on the cytosolic adaptor ASC, therefore suggesting a new pathway of cytosolic flagellin sensing. However, our work also suggested that the lack of TLR5 activity of *Brucella* flagellin and the regulation of its synthesis and/or delivery into host cells are both part of the stealthy strategy of *Brucella* towards the innate immune system. Nevertheless, as a flagellin-deficient mutant of *B. melitensis* was**

found to cause histologically demonstrable injuries in the spleen of infected mice, we suggested that recognition of FliC plays a role in the immunological stand-off between *Brucella* and its host, which is characterized by a persistent infection with limited inflammatory pathology.

Introduction

The mammalian innate immune system relies on a limited number of pattern recognition receptors (PRRs) to detect microbial-derived molecules during infection and subsequently trigger an appropriate immune response to the invading pathogen. These microbial features are often referred to as PAMPs for pathogen-associated molecular patterns. The PRRs include Toll-like receptors (TLRs), which sense PAMPs on the cell surface or in endosomes (Kawai and Akira, 2011), and nucleotide-binding domain and leucine-rich repeat containing (NLRs) proteins, which are cytosolic receptors responding to PAMPs and endogenous danger signals (Brodsky and Medzhitov, 2009; Lamkanfi and Dixit, 2009). After stimulus recognition, TLRs initiate multiple signalling pathways involved in the innate inflammatory and antimicrobial responses, as well as in the initiation and control of adaptive immune responses (Kawai and Akira, 2011). In contrast, upon stimulation, several NLRs, including NLRP1 (also known as NALP1), NLRP3 (NALP3 or cryopyrin) and NLRC4 (IpaF) assemble inflammasomes, which are multiprotein complexes responsible for activation of the inflammatory cysteine protease caspase-1 (Schroder and Tschopp, 2010).

Bacterial flagellin, the monomeric subunit of flagellar filament, is a PAMP for both systems. Extracellular flagellin is detected by TLR5 (Hayashi *et al.*, 2001) that activates the MyD88-dependent signalling pathway, leading to the nuclear translocation of NF- κ B, and the activation of mitogen-activated protein kinases (MAPK), ultimately inducing the secretion of proinflammatory cytokines and chemokines, such as IL-8 (Eaves-Pyles *et al.*, 2001; Gewirtz *et al.*, 2001; Yu *et al.*, 2003). On the other hand, flagellin injected into the cytoplasm of macrophages through bacterial virulence-associated secretion systems is sensed by NLRC4 in association with NAIP5, another member of the NLR family (Kofoed and Vance, 2011; Zhao *et al.*, 2011). Activation of caspase-1 within the

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NLRC4 inflammasome leads to the maturation and release of biologically active proinflammatory cytokines IL-1 β and IL-18 (van de Veerdonk *et al.*, 2011). Moreover, this inflammasome can trigger a proinflammatory form of cell death known as pyroptosis (Bergsbaken *et al.*, 2009). Finally, it has been shown that NLRC4 plays a role in maintaining a normal endosome–lysosome trafficking of phagocytized bacteria within macrophages (Amer *et al.*, 2006; Akhter *et al.*, 2009). There is evidence that both TLR5 and NLRC4 play a role in controlling *in vivo* infections caused by pathogenic bacteria including *Salmonella enterica* serotype Typhimurium (Feuillet *et al.*, 2006; Broz *et al.*, 2010), *Legionella pneumophila* (Hawn *et al.*, 2003; Amer *et al.*, 2006) and *Pseudomonas aeruginosa* (Feuillet *et al.*, 2006; Sutterwala *et al.*, 2007; Franchi *et al.*, 2012). However, bacterial countermeasures to avoid flagellin recognition by the innate immune system have also been described. *Helicobacter pylori* and *Campylobacter jejuni* escape TLR5 recognition as a result of changes in the amino acid sequence of flagellin (Andersen-Nissen *et al.*, 2005), and it has been suggested that *S. Typhimurium* downregulates *fliC* expression during macrophage infection to avoid a deleterious strong activation of NLRC4 inflammasome (Cummings *et al.*, 2006; Miao *et al.*, 2010a).

Brucella spp. are Gram-negative bacteria that cause brucellosis, a zoonosis of worldwide importance. In the natural reservoir hosts, including wild and domestic animals, these intracellular pathogens cause abortion and infertility. Humans are accidental hosts and *Brucella melitensis* and *Brucella abortus* are the most frequent cause of human infection (Corbel, 1997). A key characteristic of *Brucella* infection is its chronic nature. Indeed, animals can remain infected for years, and *Brucella* causes a protracted debilitating disease in untreated humans that can result in serious clinical complications (Young, 1995). As a result, brucellosis has an important economic impact on livestock and remains a major public health concern in endemic countries (Pappas *et al.*, 2006).

An important aspect of *Brucella* virulence is its capacity to survive, replicate and persist within infected cells (Atluri *et al.*, 2011). Persistence of *Brucella* within cells relies at least in part on its ability to control the intracellular trafficking of its vacuole in order to avoid lysosomal degradation and to gain access to its replicative niche derived from the endoplasmic reticulum (Anderson and Cheville, 1986). Moreover, the success of *Brucella* lies in its stealthy strategy to cope with the innate immune system. First, the structural features of the *Brucella* envelope allow it to avoid sustained recognition by PRRs and subsequent strong inflammatory responses at the onset of infection (Barquero-Calvo *et al.*, 2007). For example, *Brucella* produces a lipopolysaccharide that signals poorly through

TLR4, compared to other bacteria (Lapaque *et al.*, 2006; Barquero-Calvo *et al.*, 2007). In addition, *Brucella* can actively control the inflammatory response by producing a protein that interferes with TLR-dependent signalling pathways (Salcedo *et al.*, 2008; Radhakrishnan *et al.*, 2009; Sengupta *et al.*, 2009). Along with the lack of cytotoxicity of *Brucella* for highly parasitized host cells, all the above-mentioned features could render it less noticeable by the host innate immune system than other pathogens (Gross *et al.*, 2000; Barquero-Calvo *et al.*, 2007; Salcedo *et al.*, 2008). Nonetheless, *Brucella* spp. have virulence factors such as a VirB type IV secretion system (T4SS) (O'Callaghan *et al.*, 1999), cyclic β -1,2-glucan (Briones *et al.*, 2001; Arellano-Reynoso *et al.*, 2005) and flagellar genes (Fretin *et al.*, 2005) that are required for *Brucella* to persist within its host. Although our previous studies focused on the flagellum and its role in persistent infection, it is unknown whether *Brucella* flagellin, FliC, is sensed by the host during infection. Here, we combined host and pathogen genetic approaches to assess the potential of *Brucella* flagellin to stimulate innate immune responses.

Results

Mice fail to control infection by flagellin-deficient B. melitensis mutants

In a previous study, insertional inactivation of genes located in the three flagellar loci of *B. melitensis* was reported to result in a marked attenuation of its virulence in mice (Fretin *et al.*, 2005). At that time, it was assumed that, as described in enterobacteriaceae, the *fliC* gene was not expressed in mutants of genes encoding basal flagellar structures. However, we recently demonstrated that the flagellar expression hierarchy of *Brucella* is not conventional, as the flagellin subunit is still produced in mutants deficient in the hook or basal body (Ferooz *et al.*, 2011). To evaluate the specific impact of the absence of FliC flagellin on the virulence of *B. melitensis*, non-polar mutants of *fliC* ($\Delta fliC$) and *flbT* ($\Delta flbT$) (Ferooz *et al.*, 2011) were used to infect murine macrophages and BALB/c mice. The FliC regulator of *B. melitensis* is specifically required for the production of FliC, most likely by allowing translation of the *fliC* mRNA (Ferooz *et al.*, 2011). Accordingly, flagellin was detected neither in the $\Delta fliC$ nor in the $\Delta flbT$ strain harvested at the early exponential phase of growth, whereas the protein is produced by the isogenic wild type (wt) strain (Fig. 1A).

We first compared the intracellular growth of *B. melitensis* $\Delta fliC$ and $\Delta flbT$ to that of wt bacteria in RAW264.7 murine macrophages. No difference in colony-forming units (CFUs) was detected over a 48 h time-course (Fig. 1B). Similar results were obtained in HeLa cells (data

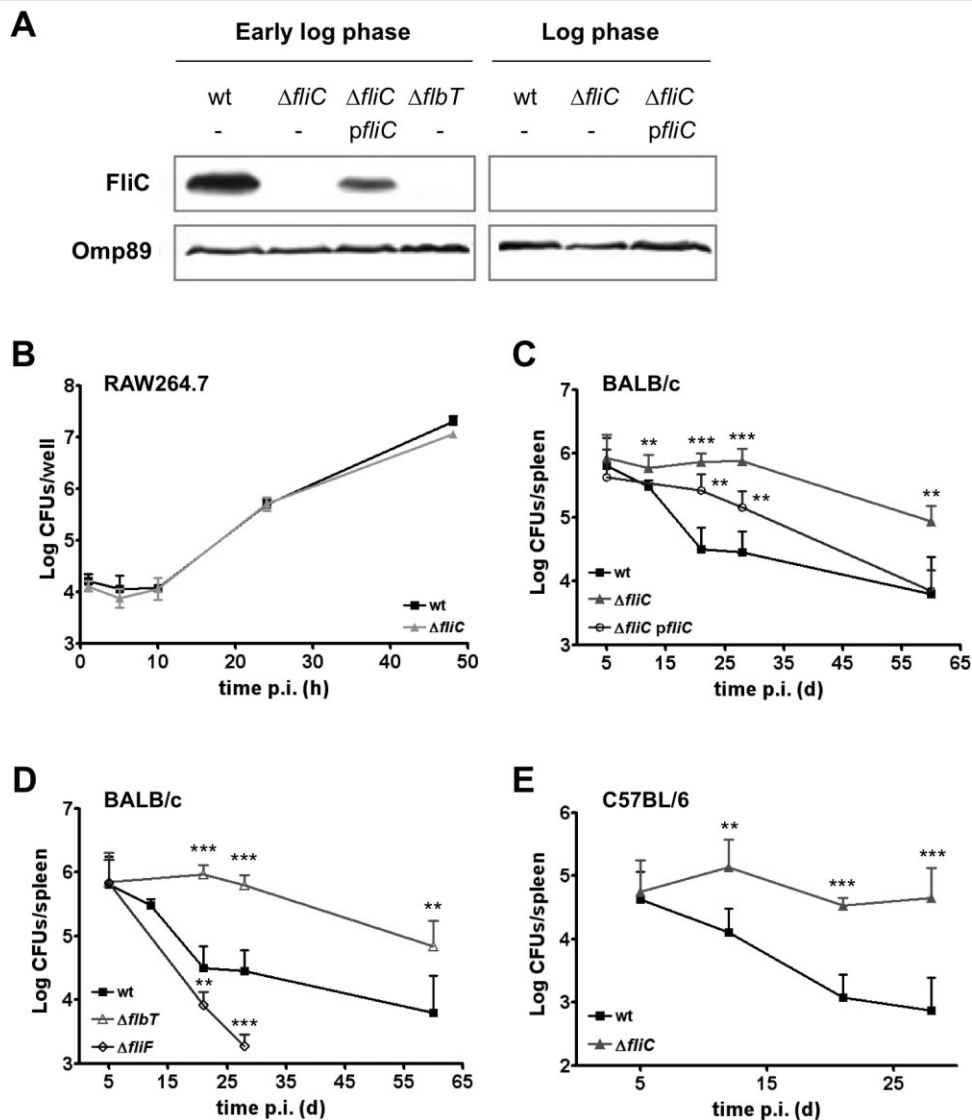


Fig. 1. Flagellin-deficient *B. melitensis* mutants infect macrophages *in vitro* with the same kinetics as wt bacteria but show enhanced persistence in mice.

A. Western blot analysis of the production of flagellin (FliC, upper panel) by *B. melitensis* strains harvested at the early log phase and the log phase of growth in 2YT rich medium. Anti-Omp89 detection was used as a loading control (lower panel). Data are representative of two independent experiments. $\Delta fliC$ p*fliC* is the complemented strain.

B. Intracellular replication of *B. melitensis* 16M wt and $\Delta fliC$ strains in RAW264.7 murine macrophages. Error bars represent the standard deviation of triplicates in one representative experiment out of three.

C–D. Infection kinetics in the spleens of wt BALB/c mice ($n = 5$) inoculated i.p. with 4×10^4 CFUs of *B. melitensis* 16M wt, $\Delta fliC$, complemented $\Delta fliC$ p*fliC*, $\Delta fliB$ T or $\Delta fliF$ strains.

E. Infection kinetics in the spleens of wt C57BL/6 mice ($n = 5$) inoculated i.p. with 4×10^4 CFUs of *B. melitensis* 16M wt or $\Delta fliC$ strains. Data represent the mean CFUs per organ and error bars represent standard deviation. Results have been analysed by ANOVA I after testing the homogeneity of variance (Bartlett). ** and *** denote highly significant ($P < 0.01$ and $P < 0.001$ respectively) differences in relation to wt infection. These results are representative of at least two independent experiments.

not shown). Consistent with a normal multiplication in endoplasmic reticulum-derived vacuoles, both $\Delta fliC$ mutant and its isogenic parental strain were found to replicate within calnexin-positive compartments of HeLa cells at 24 h post infection (p.i.) (data not shown).

Despite the absence of an obvious role for *Brucella* flagellar genes in cellular models of infection, several

reports have shown that they are required for the establishment of a persistent infection *in vivo* (Fretin *et al.*, 2005; Zygmunt *et al.*, 2006). To re-evaluate the role of flagellar proteins *in vivo*, BALB/c mice were infected via the intraperitoneal route with *B. melitensis* 16M $\Delta fliC$, $\Delta fliB$ T and $\Delta fliF$ non-polar mutants. None of the mutants was significantly attenuated 5 days p.i., as compared with

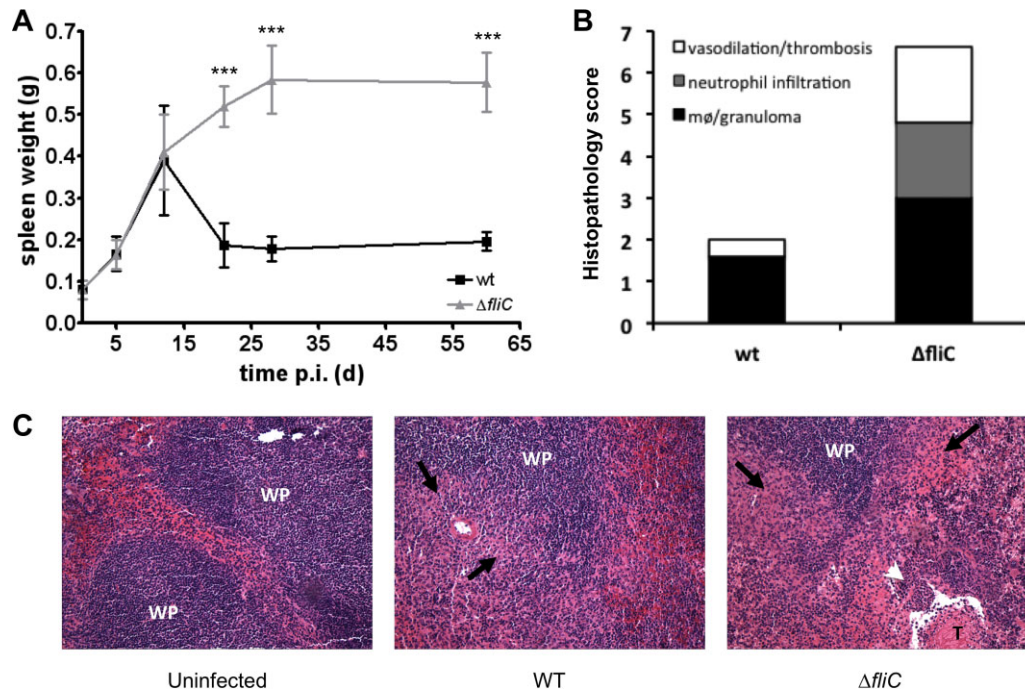


Fig. 2. Enhanced persistence of *B. melitensis* $\Delta fliC$ in mice is associated with increased pathology.

A. Kinetics of splenomegaly in wt female BALB/c mice ($n = 5$) injected i.p. with 4×10^4 CFUs of wt or $\Delta fliC$ strains of *B. melitensis* 16M. Data represent the mean spleen weight and error bars represent standard deviation. Results have been analysed by ANOVA I after testing the homogeneity of variance (Bartlett). *** denotes highly significant ($P < 0.001$) differences in relation to wt infection.

B. Splenic pathology caused by a 28-day infection was determined using the histopathology scoring system as described in *Experimental procedures*. Data were analysed using a Mann–Whitney test, and the mean histopathology scores were significantly different ($P = 0.009$).

C. Representative photomicrographs ($\times 10$) of histopathology of spleens from BALB/c mice uninfected or infected for 28 days with *B. melitensis* wt or $\Delta fliC$ strain. WP, white pulp; T, thrombosis; black arrows, granuloma; white arrowhead, neutrophil infiltration. These results are representative of at least two independent experiments.

the parental strain (Fig. 1C and D). Moreover, we could confirm that the basal body protein FliF is required for full virulence. Indeed, the $\Delta fliF$ mutant was attenuated at 3 and 4 weeks p.i. (Fig. 1D). In contrast, the virulence of the $\Delta fliC$ strain was exacerbated when compared to its isogenic parental strain, as $\Delta fliC$ -infected mice presented a higher bacterial load in the spleen from 12 days until 60 days p.i. (Fig. 1C). A higher bacterial count was also observed at the same times in the livers of mice infected with the $\Delta fliC$ mutant (data not shown). Similarly, an enhanced persistence of the $\Delta fliC$ strain in the spleens of the resistant C57BL/6 mice has also been observed (Fig. 1E). The use of a low-copy plasmid carrying *fliC* gene along with its predicted flanking regulatory sequences, which restores regulated production of flagellin in the $\Delta fliC$ strain (Fig. 1A), allowed partial complementation of the phenotype of the newly constructed $\Delta fliC$ mutant at 28 days p.i. and full complementation at 60 days p.i. (Fig. 1C). Moreover, we could show that the $\Delta fliB$ mutant had similar infection kinetics than the $\Delta fliC$ strain in the spleen of BALB/c mice (Fig. 1D). This further supports the fact that the apparent inability of the host to control bacterial infection is specifically due to the lack of flagellin production by *Brucella*.

Mice infected with B. melitensis $\Delta fliC$ mutant exhibit severe splenic pathology

Brucella is known to induce splenomegaly in infected hosts. During the course of a *B. melitensis* 16M infection in BALB/c mice, the spleen weight increases and peaks around 0.4 g (fourfold the spleen weight of an uninfected mice) at 12 days p.i. Afterwards, the spleen weight decreases but remains twice the normal value until the end of the experiment (Fig. 2A). In contrast, we found that the splenomegaly of mice infected with flagellin-deficient mutants, while displaying kinetics similar to those of the wt infection during the first 12 days, continued to increase until 28 days p.i. and reached a plateau of almost five or six times the normal spleen weight by the end of the experiment (Fig. 2A for $\Delta fliC$, data not shown for $\Delta fliB$). A similar exacerbation of splenomegaly was also observed in C57BL/6 mice at 21 days p.i. with the $\Delta fliC$ mutant (data not shown). This was in accordance with the enhanced persistence of the flagellin-deficient mutants in mice (Fig. 1C–E).

We further examined the splenic histopathology of BALB/c mice infected for 28 days with wt or $\Delta fliC$ *B. melitensis* strain. At this time, mice infected with the

$\Delta fliC$ strain showed a markedly exacerbated splenic inflammation characterized by increased vasodilation, thrombosis, neutrophil infiltration and granuloma formation (Fig. 2B and C). In contrast, mice infected for 28 days with wt *B. melitensis* had nearly normal splenic morphology, as compared with non-infected mice.

Ectopic production of flagellin attenuates the virulence of *B. melitensis* in vivo

Mice apparently fail to control infection caused by *B. melitensis* 16M $\Delta fliC$ or $\Delta flbT$ at late time points. This observation suggests that production of flagellin by *Brucella* somehow influences the course of infection. To further test this hypothesis, we engineered a *B. melitensis* 16M strain, designated *BruFliC^{ON}*, that constitutively expresses a plasmid-encoded copy of *fliC* from *Escherichia coli* *Plac*. Western blot analysis confirmed that, while production of flagellin by wt bacteria is only detectable at the early exponential phase of growth, *BruFliC^{ON}* produced higher levels of flagellin throughout *in vitro* growth (Fig. 3A). Ectopic production of flagellin did not impair the invasion and replication abilities of *Brucella* in macrophages *in vitro* (Fig. 3B). However, we found that the *BruFliC^{ON}* strain was attenuated *in vivo* compared with wt *B. melitensis* 16M. While no difference in splenic bacterial load was observed between the two strains at 5 days p.i. of BALB/c mice, 0.5 to 1 log fewer CFU of *BruFliC^{ON}* bacteria were recovered at 12, 21 and 28 days p.i. (Fig. 3C). Reduced colonization of *BruFliC^{ON}* was also observed in the liver of infected BALB/c, and similar results were also obtained with C57BL/6 mice (data not shown).

Brucella flagellin lacks TLR5 agonist activity

The altered virulence of the $\Delta fliC$ and *BruFliC^{ON}* mutants led us to hypothesize that *Brucella* flagellin is detected by the host in order to mount a protective immune response. To ascertain whether innate immune sensing of flagellin contributes to enhanced control of systemic *Brucella* infection, we first determined whether *Brucella* flagellin possesses agonist activity for TLR5. To this end, epitope-tagged FliC flagellins from *Brucella* (*BruFliC*-FLAG) or *S. enterica* serotype Typhimurium (*S. Typhimurium*; StFliC-FLAG) were expressed in an *S. Typhimurium* *fliCfljB* mutant (EHW26) lacking endogenous flagellin expression. Immunoblotting with the anti-FLAG antibody demonstrated that both *BruFliC*-FLAG and StFliC-FLAG were secreted to the supernatant in similar amounts (Fig. 4A). Addition of the C-terminal FLAG tag to StFliC prevents its assembly into flagellar filaments, thereby allowing for a direct comparison of effects of flagellin monomers in the absence of a confounding effect on

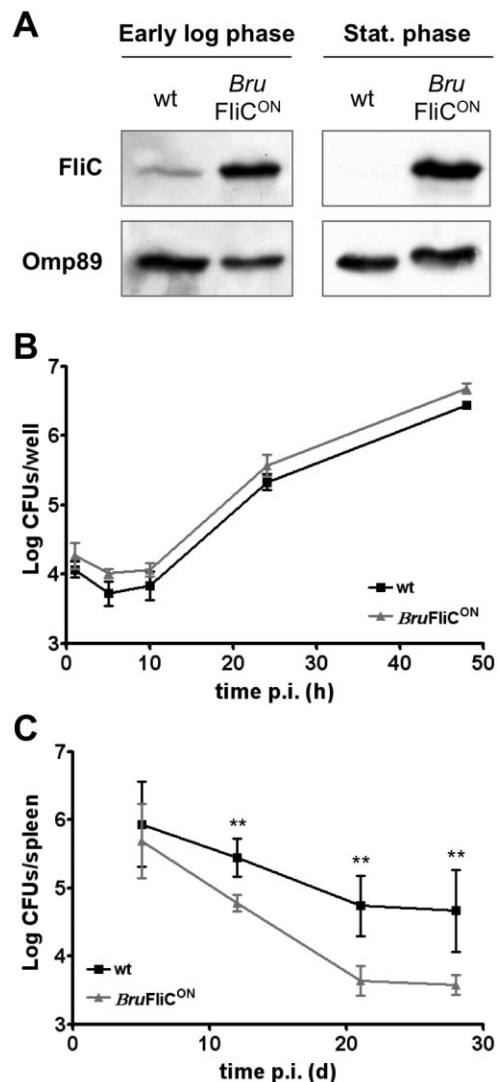


Fig. 3. Constitutive production of flagellin does not impair replication of *B. melitensis* 16M in macrophages *in vitro*, but attenuates its virulence *in vivo*.

A. Western blot analysis of flagellin (FliC, upper panel) production in wt and *BruFliC^{ON}* strains during early exponential and stationary phases of growth in 2YT rich medium. Detection of Omp89 was used as a loading control.

B. Intracellular replication of wt and *BruFliC^{ON}* strains in RAW264.7 murine macrophages. Error bars represent the standard deviation of triplicates in one representative experiment out of two.

C. Infection kinetics in the spleens of wt BALB/c mice ($n = 5$) inoculated i.p. with 4×10^4 CFUs of wt or *BruFliC^{ON}* strain. Data represent the mean CFUs per organ and error bars represent standard deviation. Results have been analysed by ANOVA I after testing the homogeneity of variance (Bartlett). ** denotes highly significant ($P < 0.01$) difference in relation to wt infection. These results are representative of at least two independent experiments.

motility, as strains expressing either StFliC-FLAG or *BruFliC*-FLAG were aflagellate and non-motile (data not shown).

Culture supernatants of *S. Typhimurium* *fliCfljB* expressing recombinant flagellins were used to treat two

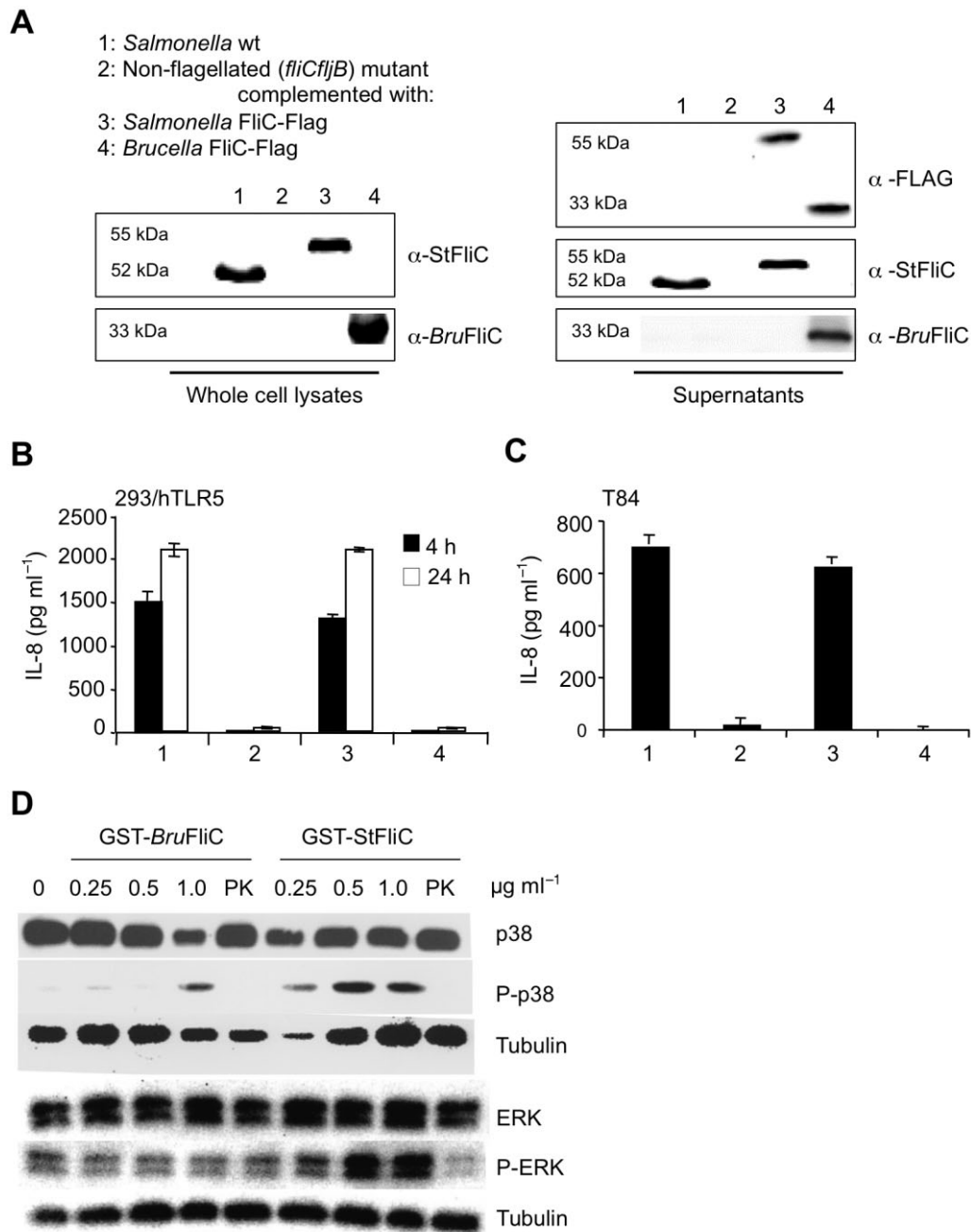


Fig. 4. *Brucella* flagellin lacks TLR5 agonist activity.

A–C. FLAG-tagged flagellins from *S. enterica* serotype Typhimurium (StFliC) or *B. abortus* (*Bru*FliC) were expressed in an *S. Typhimurium* *fliCfljB* mutant, and culture supernatants containing recombinant flagellins were used to treat cells. (A) Western blot showing production of bacterium-associated flagellins from *S. Typhimurium* wt (lane 1), *S. Typhimurium* *fliCfljB* mutant (lane 2), *fliCfljB* mutant expressing StFliC-FLAG (lane 3) or *fliCfljB* mutant expressing *Bru*FliC-FLAG (lane 4). Flagellins were detected both in the pellets (left panel) and in the concentrated supernatants (right panel) of *S. Typhimurium* strains. Thirty nanograms of concentrated supernatant proteins from *S. Typhimurium* strains expressing recombinant flagellins were used to treat HEK293/hTLR5 cells for 4 or 24 h (B) and T84 cells for 8 h (C). IL-8 in cell supernatants was measured by ELISA.

D. Activation of p38 and ERK MAPK in T84 cells by purified recombinant flagellins from *Brucella* (GST-*Bru*FliC) and *S. Typhimurium* (GST-*St*FliC) was measured by Western blot analysis with anti-p38, anti-phosphorylated (P-)p38, anti-ERK and anti-P-ERK. Detection of tubulin was used as a loading control. Purified flagellins treated with proteinase K (PK) were used as a control. All data shown are from an individual experiment that was repeated at least twice with similar results.

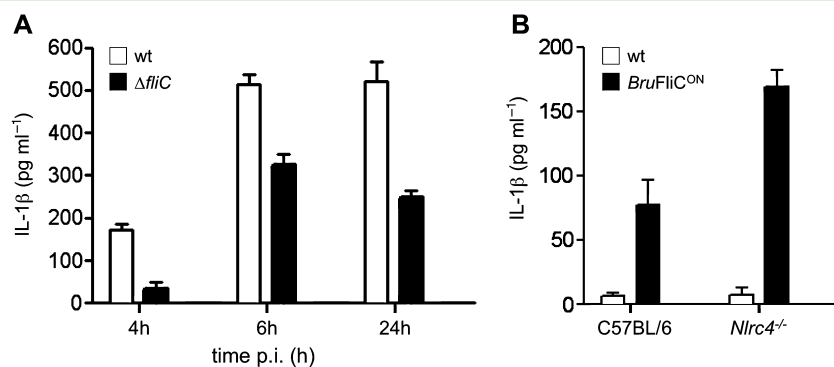


Fig. 5. *B. abortus* flagellin induces IL-1 β in an NLRC4-independent manner.

A. Primary bone marrow-derived macrophages from C57BL/6 mice were primed with LPS and inoculated with *B. melitensis* 16M wt or the Δ *fliC* mutant and IL-1 β was measured in the culture supernatants by ELISA. Results are shown as the mean \pm standard deviation of data from an individual experiment that was repeated four times with similar results.

B. Immortalized, LPS-primed C57BL/6 or *Nlrc4*^{-/-} bone marrow-derived macrophages were inoculated with *B. melitensis* 16M wt or the *BruFliC*^{ON} strain. IL-1 β in the supernatant was measured at 6 h after inoculation. Data shown are combined from three independent experiments with triplicate samples, and represent the mean \pm standard deviation of all data.

TLR5-expressing cell lines: HEK293/hTLR5 and the colonic epithelial cell line T84 (Fig. 4B and C). Both cell lines secreted interleukin 8 (IL-8) on infection with strains expressing native or FLAG-tagged StFliC, demonstrating that addition of the epitope tag to the C-terminus of flagellin did not affect its TLR5 agonist activity. Stimulation of IL-8 secretion was dependent on flagellin in both cell lines, as culture supernatants from the *fliCfljB* mutant elicited little (Fig. 4C) or no (Fig. 4B) IL-8. In contrast to StFliC-FLAG, expression of *BruFliC*-FLAG did not elicit IL-8 secretion above the level of the *fliCfljB* mutant. Similar results were obtained when T84 or HEK293/hTLR5 cells were infected with *S. Typhimurium* strains expressing recombinant flagellins (data not shown). The response to *BruFliC* did not appear to be delayed, as extending the time of the assay to 24 h did not allow detection of a response comparable to that elicited by StFliC-FLAG (Fig. 4B). As a second readout for TLR5 signalling, we assayed activation of MAPK p38 and ERK by treatment with purified, GST-tagged flagellins. Phosphorylation of both p38 and ERK was induced to a greater extent by GST-StFliC than by GST-*BruFliC*, and notably no increase in phosphorylation of ERK could be detected after treatment with GST-*BruFliC* (Fig. 4D). Taken together, these results demonstrate that compared to *S. Typhimurium* flagellin, the ability of *Brucella* flagellin to stimulate TLR5 signalling is greatly reduced.

Cytosolic sensing pathways detect *Brucella* flagellin during infection of macrophages

In addition to TLR5, flagellin that enters the cytosol of host macrophages can be sensed by the NLRC4/NAIP5 pathway (Kofoed and Vance, 2011; Zhao *et al.*, 2011). To

determine whether cytosolic pathways could detect flagellin during *Brucella* infection, we first used the TEM-1 β -lactamase assay to detect translocation of flagellin into the cytosol of *B. abortus*-infected J774 macrophage-like cells. For these experiments, J774 cells were infected with *B. abortus* 2308 expressing either a C-terminally tagged copy of *Brucella* flagellin or an irrelevant protein (GST), from a multi-copy plasmid (pFlagTEM-1; Sun *et al.*, 2007). While cells infected with *B. abortus* expressing GST::Flag-TEM-1 showed no cytosolic β -lactamase activity (no β -lactamase-positive cells in four experiments), 0.94% (range: 0.3–2.1%) of cells infected with *B. abortus* expressing the flagellin fusion protein were β -lactamase-positive, suggesting potential access of low amounts of flagellin to the cytosol of *Brucella*-infected cells. Next, we determined whether, in primary macrophages, cytosolic flagellin could stimulate innate immune responses. To this end, we compared the ability of *B. melitensis* and its isogenic Δ *fliC* mutant to elicit IL-1 β secretion from primary bone marrow-derived macrophages (BMDM). Compared to *B. melitensis* wt, the Δ *fliC* mutant elicited significantly reduced IL-1 β secretion (Fig. 5A). This reduction was not the result of differing numbers of intracellular bacteria of the Δ *fliC* mutant, as both the Δ *fliC* mutant and wt *B. melitensis* were present in the same numbers (data not shown). This partial reduction in IL-1 β secretion suggests that recognition of flagellin contributes to activation of the caspase-1 inflammasome.

The mechanism of cytosolic flagellin sensing in the context of intracellular infection was further investigated using the *B. melitensis* FliC^{ON} strain, which expresses flagellin constitutively. This strain, as well as a control carrying the empty plasmid pBBR1MCS, was used to infect immortalized BMDM from mice deficient in NLRC4

(Fig. 5B). Constitutive expression of FliC did not affect the ability of *B. melitensis* to survive intracellularly (Fig. 3B and data not shown). As in LPS-primed BMDM the amount of IL-1 β was maximal at 6 h after infection with *Brucella* (Fig. 5A), we looked at the IL-1 β response only at this time point. The *BruFliC*^{ON} strain elicited significantly more IL-1 β secretion than the control strain (Fig. 5B), confirming data shown in Fig. 5A. While these results suggested that under conditions of flagellin expression, flagellin can be sensed by cytosolic PRRs that lead to activation of caspase-1 and secretion of IL-1 β , NLRC4 was not required for flagellin-dependent stimulation of IL-1 β secretion by BMDM *in vitro* (Fig. 5B).

Brucella flagellin elicits IL-1 β secretion by a mechanism that is distinct from the NLRC4/NAIP5 pathway

As *B. melitensis* is known to inhibit innate immune signaling (Salcedo *et al.*, 2008; Radhakrishnan *et al.*, 2009; Sengupta *et al.*, 2009), we determined whether purified flagellin, in the absence of other *Brucella* factors, would signal similarly to flagellin expressed during cellular infection. For this purpose, purified GST-*BruFliC* and GST-*StFliC* were introduced into the cytosol of BMDM using the cationic lipid DOTAP (Franchi *et al.*, 2006). Both *BruFliC* and *StFliC* elicited dose-dependent secretion of IL-1 β from BMDM from C57BL/6 mice when introduced into the cytosol using DOTAP (Fig. 6A). Neither GST, DOTAP alone, nor recombinant flagellins in the absence of DOTAP elicited any secretion of IL-1 β (Fig. 6A and data not shown). Comparison of IL-1 β secreted in response to equal amounts of *StFliC* or *BruFliC* suggested that the proinflammatory activity of *StFliC* was slightly higher than that of *BruFliC* (Fig. 6A). Secretion of IL-1 β in response to *S. Typhimurium* FliC was dependent on NLRC4 and only partially dependent on the adaptor protein ASC (apoptosis-associated speck-like protein), as reported previously (Broz *et al.*, 2010). In contrast, *BruFliC* elicited IL-1 β secretion that required ASC, but was independent of NLRC4, at least in cultured BMDM (Fig. 6B and C). These results suggested that in BMDM, *Brucella* flagellin was sensed by a cytosolic mechanism that differs from the NLRC4/NAIP5-dependent response to *S. Typhimurium* FliC (Kofoed and Vance, 2011; Zhao *et al.*, 2011).

The cytosolic flagellin-detection pathway is implicated in the control of B. melitensis infection in vivo

To evaluate the potential impact of caspase-1 inflammasomes on the control of *Brucella* infection *in vivo*, we infected *Nlrc4*^{-/-} and *Casp1*^{-/-} C57BL/6 mice with *B. melitensis* 16M. Splenic bacterial count was examined 21 days p.i., a time at which wt mice manage to effectively control infection caused by flagellin-producing *Brucella*

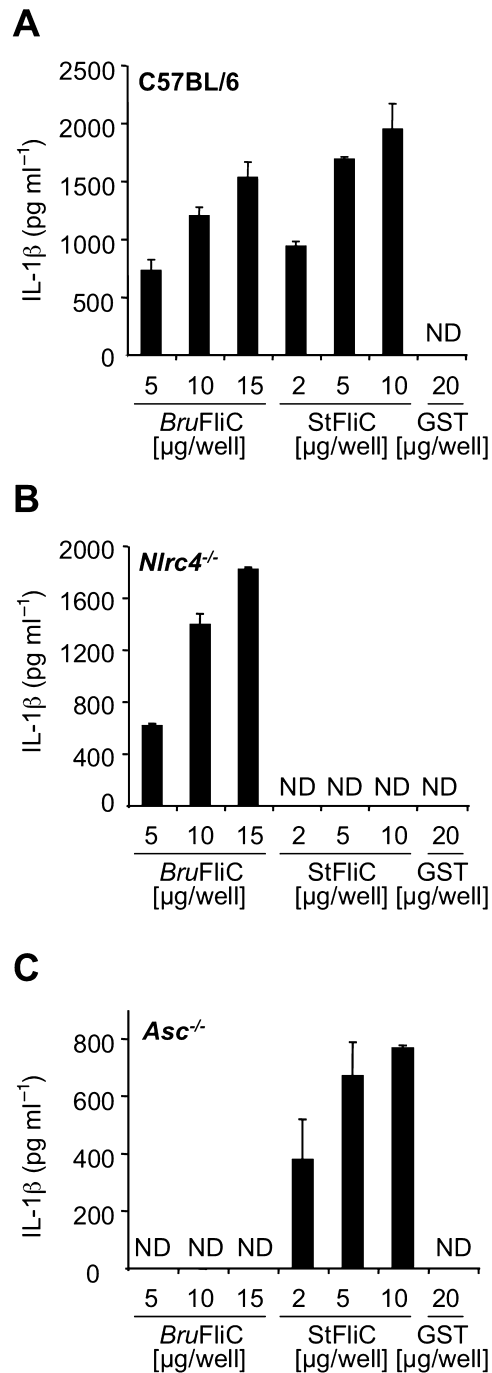


Fig. 6. Introduction of recombinant *Brucella* flagellin into the host cell cytosol results in ASC-dependent, but NLRC4-independent secretion of IL-1 β . Graded amounts of GST-*BruFliC* and GST-*StFliC* fusion proteins were delivered to the cytosol of LPS-primed primary bone marrow-derived macrophages from C57BL/6 (A), *Nlrc4*^{-/-} (B) or *Asc*^{-/-} (C) mice, using the cationic lipid DOTAP. Treated macrophages were incubated for 3 h before measurement of IL-1 β in the supernatants by ELISA. Results are expressed as the mean of triplicate samples, with error bars representing the range of the data from one of two independent experiments with the same outcome.

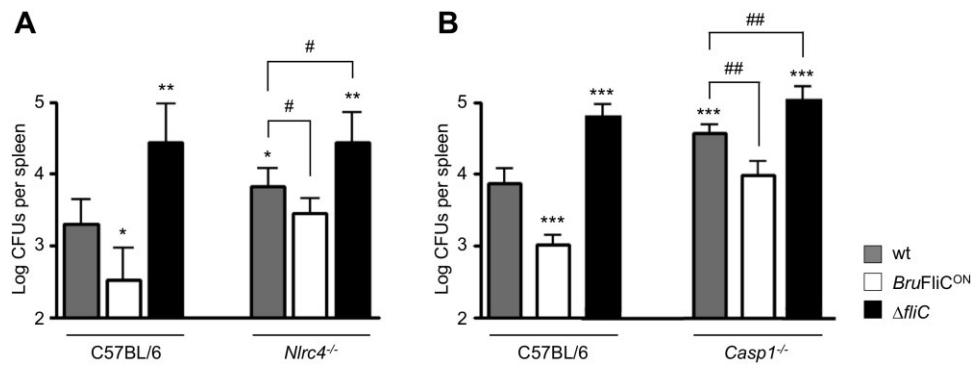


Fig. 7. NLRC4 inflammasome is implicated in the control of *B. melitensis* infection *in vivo*. Wild type, *Nlrc4*^{-/-} (A) and *Casp1*^{-/-} (B) C57BL/6 mice ($n = 5$) were injected i.p. with 4×10^4 CFUs of *B. melitensis* wt, *BruFliC*^{ON} or $\Delta fliC$ strain, as indicated in the figure. Mice were sacrificed 21 days post infection and CFUs per spleen were determined. These results are representative of at least two independent experiments. Data have been analysed by ANOVA I after testing the homogeneity of variance (Bartlett). *, ** and *** denote significant ($P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively) differences in relation to C57BL/6 wt infection by wt bacteria. # and ## denote significant ($P < 0.05$ and $P < 0.01$ respectively) differences in relation to knock-out mice infection by wt bacteria.

strains (Figs 1C–E and 3C). At this time, we observed that NLRC4 (Fig. 7A) and caspase-1 (Fig. 7B) deficiency moderately but significantly affected the resistance of mice to infection. This suggests that the NLRC4–caspase-1 axis is required for the host to control *B. melitensis* 16M infection, possibly through recognition of cytosolic flagellin. To further test this hypothesis, the *BruFliC*^{ON} strain was used to infect *Nlrc4*^{-/-} and *Casp1*^{-/-} mice. As shown previously, virulence of this strain is attenuated compared to wt *B. melitensis* 16M, as the spleen of *BruFliC*^{ON}-infected C57BL/6 mice contained less CFUs than those infected by the wt strain (Fig. 7). Interestingly, this virulence defect was rescued in mice deficient for the cytosolic flagellin sensor NLRC4 (Fig. 7A) or the downstream caspase-1 (Fig. 7B). These data indicate that, in contrast to what has been observed *in vitro* (Figs 5B and 6B), *Brucella* flagellin can activate the NLRC4 inflammasome *in vivo*. Nevertheless, we found that the *BruFliC*^{ON} strain was still attenuated (a significant 0.5 log decreased CFUs in the spleen) compared to *B. melitensis* 16M wt in *Nlrc4*^{-/-} and *Casp1*^{-/-} mice 21 days p.i. (Fig. 7). This suggests that both inflammasome-dependent and inflammasome-independent control of infection operates downstream detection of *Brucella* flagellin *in vivo*. This hypothesis is further supported by the finding that although *Nlrc4*^{-/-} and *Casp1*^{-/-} mice infected with wt *B. melitensis* 16M had significantly higher splenic bacterial counts than those of wt mice, it remained significantly lower than those of mice infected with the $\Delta fliC$ mutant (Fig. 7).

B. melitensis $\Delta fliC$ mutant fails to elicit early granuloma formation in the spleen of infected mice

Chronic granulomatous inflammation in the spleen of natural hosts, humans and mice is the hallmark of *Brucella* infection (Spink *et al.*, 1949; Enright *et al.*, 1990).

Recently, we revealed the pivotal role of early splenic granuloma formation in the ability of mice to control bacterial dissemination (Copin *et al.*, 2012). Here, we used a rabbit polyclonal serum raised against *B. melitensis* (anti-*Bru*) with the aim to compare the distribution of putative infected cells in the spleen of BALB/c mice inoculated with *B. melitensis* 16M wt or $\Delta fliC$ strain. Five days after infection with *B. melitensis* 16M wt, clusters of cells stained with anti-*Bru* (*Bru*-positive cells) were found equally in white pulp and red pulp area of the spleen (Fig. 8). These clusters consisted primarily of CD11b⁺ cells, suggesting that they corresponded to the granuloma previously described (Copin *et al.*, 2012). Strikingly, at the same time, the number of *Bru*-positive cell clusters counted in splenic sections of $\Delta fliC$ -infected mice was reduced (Fig. 8). This apparent defect in early splenic granuloma formation suggests the importance of flagellin sensing by the host for the orchestration of this typical tissue response to *Brucella* infection.

Discussion

Intracellular survival and immune evasion both contribute to persistence of *Brucella* in the host (Atluri *et al.*, 2011). Recent studies have shown that *Brucella* uses passive as well as active mechanisms to evade detection by TLRs of the innate immune system (Lapaque *et al.*, 2006; Barquero-Calvo *et al.*, 2007; Salcedo *et al.*, 2008; Radhakrishnan *et al.*, 2009; Sengupta *et al.*, 2009). Accordingly, the inflammatory response induced at the onset of *Brucella* infection is lower than observed with pyogenic infections such as salmonellosis (Barquero-Calvo *et al.*, 2007). Actually, brucellae are not entirely invisible to the immune system, which can still detect them and shape a Th1 response to control infection (Murphy *et al.*, 2001; Copin *et al.*, 2007). However, the host immune response

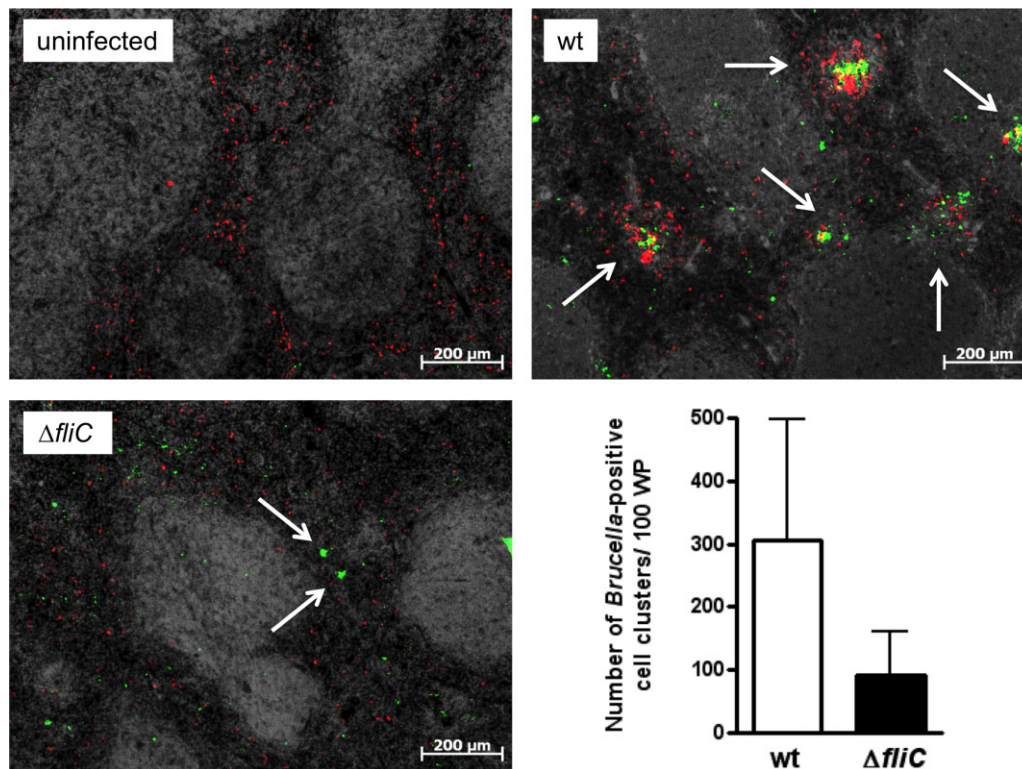


Fig. 8. The distribution of *Bru*⁺ cells is different in the spleen of mice infected by the $\Delta fliC$ mutant, compared to wt infection. Localization of *Bru*⁺ cells (green) and CD11b⁺ cells (red) in the spleen of BALB/c mice non-infected or infected with *B. melitensis* wt or the $\Delta fliC$ strain. The graph represents the relative number of clusters of *Bru*⁺ cells. Errors bars are the standard deviation calculated on countings of four mice from two independent experiments.

is not sufficient to eliminate bacteria, resulting in a chronic state of infection characterized by a balance between pathogen virulence and host resistance. The impact of *Brucella* flagellin on infection had not been reported yet. The data presented here suggest that flagellin plays a crucial role in the interplay between *Brucella* and its host, as its detection by the innate immune system is required for the control of infection *in vivo*, although some characteristics of *Brucella* flagellin would contribute to the stealthy strategy of this pathogen.

The use of two mutants of *B. melitensis* 16M that either overproduce or lack the FliC flagellin has shown that this protein hinders bacterial replication *in vivo*. Indeed, a strain engineered to ectopically produce flagellin (*Bru*FliC^{ON}) was attenuated in mice, whereas deletion of *fliC* ($\Delta fliC$) enhanced persistence of *B. melitensis* 16M in these conditions. Our *in vivo* data are consistent with studies reporting exacerbated infections caused by a flagellin-deficient mutant of *Salmonella enterica* serovar Typhimurium (Vijay-Kumar *et al.*, 2006), *L. pneumophila* (Molofsky *et al.*, 2006) or *Pseudomonas syringae* pv. *Tabaci* (Li *et al.*, 2005), as well as virulence attenuation due to flagellin overproduction by *S. Typhimurium* (Salazar-Gonzalez *et al.*, 2007; Miao *et al.*, 2010a) and *Listeria monocytogenes* (Grundling *et al.*, 2004). These

findings also suggest that *Brucella* flagellin is an important immune target during infection, and our work provides first insight into the mechanisms involved.

TLR5 and the NLRC4/NAIP5 complex are the only proteins currently known as innate immune sensors of extracellular and cytoplasmic bacterial flagellin respectively (Miao *et al.*, 2007).

In agreement with a recent paper quoting that purified *Brucella* flagellin does not induce expression of interferon-inducible resistance proteins (IRGs) in murine macrophages (Lapaque *et al.*, 2009), the data reported in this paper allow us to conclude that *Brucella* flagellin is not a TLR5 agonist. This is consistent with its atypical sequence as it lacks the amino acid residues required to stimulate this PRR (Andersen-Nissen *et al.*, 2005). Thus, we propose that *Brucella* evades TLR5-mediated detection, and that it could be part of its stealthy strategy to avoid activation of the innate immune system during the onset of infection.

Cytosolic flagellin activates a complex comprising the NLR family proteins NLRC4 and NAIP5 (Franchi *et al.*, 2006; Miao *et al.*, 2006; Kofoed and Vance, 2011; Zhao *et al.*, 2011). This complex senses a highly conserved region of the C-terminal part of the flagellin critical for flagellum filament assembly (Yonekura *et al.*, 2003), but

that is required neither for flagellin translocation into the host cell cytosol nor for TLR5 activation (Lightfield *et al.*, 2008). The C-terminal 35 amino acid residues are conserved in *Brucella* FliC flagellin, as they share respectively 46% and 40% identity with *L. pneumophila* FlaA and *S. Typhimurium* FliC, both known to activate NLRC4 (Franchi *et al.*, 2006; Zamboni *et al.*, 2006) and sharing themselves 60% identity. Recently, it has been proposed that the minimal motif of flagellin sensed by NLRC4 comprises the highly conserved last C-terminal residues VLSLL found in *L. pneumophila* FlaA and *S. Typhimurium* FliC (Lightfield *et al.*, 2008; Miao *et al.*, 2010b). This motif is semi-conserved in *Brucella* flagellin that bears an ILSFR motif.

Our results suggest that, similar to what is seen with *L. pneumophila* infection (Amer *et al.*, 2006; Case *et al.*, 2009), the NLRC4–caspase-1 axis is involved in the control of *B. melitensis* 16M *in vivo* (Fig. 7). However, the absence of NLRC4 or caspase-1 stimulation in mice infected with the flagellin-deficient *B. melitensis* 16M $\Delta fliC$ or $\Delta flbT$ mutants cannot by itself account for the inability of the host to control infection. Indeed, the relative differences of virulence between *B. melitensis* 16M wt and $\Delta fliC$ strains were only partially reduced in *Nlrc4*^{-/-} and *Casp1*^{-/-} mice (Fig. 7), indicating involvement of both NLRC4/caspase-1-dependent and independent mechanisms in the control of *Brucella* downstream flagellin recognition. This contrasts with what is observed after intratracheal infection of mice with *L. pneumophila*. Indeed, in this case, the number of *flaA* mutants and wt bacteria in the lungs of *Nlrc4*^{-/-} and *Casp1*^{-/-} is similar (Amer *et al.*, 2006; Case *et al.*, 2009). Therefore, it suggests that *Brucella* flagellin is an immune target not only for the cytosolic sensor NLRC4 *in vivo*. The slight attenuation of the *BruFliC*^{ON} strain compared to *B. melitensis* 16M wt in *Nlrc4*^{-/-} and *Casp1*^{-/-} mice 21 days p.i. (Fig. 7) is consistent with the hypothesis that *Brucella* flagellin stimulates another immune pathway in addition to the NLRC4/caspase-1 axis. The ASC-dependent signalling suggested by our *ex vivo* data (Fig. 6) could be this additional pathway. *Brucella* FliC is the first flagellin found to induce IL-1 β secretion from macrophages *in vitro* in an NLRC4-independent manner. Whether other poor agonists of TLR5 such as flagellins from ϵ -Proteobacteria activate this uncommon pathway remains to be determined.

Activation of innate immune pathways by flagellin would play a role in limiting replication of *Brucella in vivo*. Interestingly, we found that the lack of flagellin affected the control of *B. melitensis* infection by both susceptible BALB/c and resistant C57BL/6 mice (Fig. 1C–E). BALB/c mice are known for their intrinsic reduced capacity to mount a Th1 immune response and are subsequently less able to control *Brucella* infections than C57BL/6

mice, having notably an increased bacterial load in the spleens during the plateau phase (Fernandes *et al.*, 1996; Sathiyaseelan *et al.*, 2006; Copin *et al.*, 2007; Vitry *et al.*, 2012). The hypervirulence of the $\Delta fliC$ mutant in BALB/c and C57BL/6 mice suggests that immune detection of flagellin *in vivo* activates one or several immune effector mechanisms that are shared by both mouse species and that are critical for the control of *Brucella* infection. The immune effector mechanisms triggered by flagellin detection during *Brucella* infection remain to be uncovered. Processing of the proinflammatory cytokines pro-IL-1 β and pro-IL-18 (Raupach *et al.*, 2006; Dinarello, 2009), pyroptosis (Bergsbaken *et al.*, 2009; Miao *et al.*, 2010a) and control of phagosome maturation (Amer *et al.*, 2006; Akhter *et al.*, 2009) that can all result from caspase-1 activation are important processes for innate immunity against bacterial pathogens (Brodsky and Monack, 2009).

Besides its impact on the innate immune system, it is known that bacterial flagellin is also a target of the adaptive immune response (Salazar-Gonzalez and McSorley, 2005). However, whether the adaptive immune system responds to MHC class II-presented flagellin peptides during infection by *Brucella* is currently not known.

While searching for immune effector mechanisms triggered by flagellin detection and involved in the control of *Brucella* replication in mice, we found that the $\Delta fliC$ mutant fails to elicit early granulomatous response in the spleen of mice infected for 5 days, a time at which the mutant is found at a similar level as the wt strain (Fig. 8). Thus, we suggest that detection of flagellin by the host would play a role in early granuloma development during brucellosis. Although the granulomatous response was stronger at 28 days p.i. (Fig. 2B), when the $\Delta fliC$ strain colonized spleens at higher extent than wt, an early alteration in this response could contribute to the apparent failure of mice to control infections caused by the flagellin-deficient mutants of *B. melitensis* 16M. Indeed, granulomatous inflammation is the typical tissue response to *Brucella* infection in both mice and humans (Spink *et al.*, 1949; Hunt and Bothwell, 1967; Enright *et al.*, 1990), and a recent study has demonstrated the crucial role of early formation of splenic granuloma in the control of *B. melitensis* 16M (Copin *et al.*, 2012). Whether granuloma formation during infection by *Brucella* depends on ASC, NLRC4 and/or caspase-1 is currently unknown. Up to now, a role for the NLRC4 inflammasome in such a response has never been reported. However, it was recently shown that granuloma formation in chronic *Mycobacterium tuberculosis* infection is dependent on ASC, whereas it does not require caspase-1 (McElvania Tekippe *et al.*, 2010).

S. Typhimurium translocates flagellin from its containing-vacuole into the cytosol of infected cells by a

SPI1-T3SS-dependent but flagellar secretory apparatus-independent process (Sun *et al.*, 2007). Similarly, a Dot/Icm T4SS-mediated flagellin translocation has been suggested in the case of *L. pneumophila* (Molofsky *et al.*, 2006; Ren *et al.*, 2006). Here, we show that *Brucella* flagellin is also translocated into the host cell cytosol. Interestingly, flagellin translocation was not seen when a *virB2* mutant was used to infect macrophages (data not shown), suggesting that VirB T4SS may play a role in flagellin translocation. Interestingly, a requirement for the T4SS to elicit splenic microgranuloma formation has been proposed (Rolan *et al.*, 2009). According to our results, it could be envisioned that the VirB T4SS of *Brucella* elicits a granulomatous response by translocating flagellin. However, as the T4SS is also essential for *Brucella* to reach its replicative niche (Celli *et al.*, 2003), additional studies would be necessary to determine whether the role of the T4SS in release of flagellin to the host cytosol is direct or indirect. The TEM-1 β -lactamase reporter assay has been previously used to demonstrate translocation of *S. Typhimurium* flagellin into the cytosol of infected macrophages (Sun *et al.*, 2007). We observed that the amount of flagellin translocated into cells by *Brucella* is far less than by *Salmonella*. While flagellin could be detected in the cytosol of 77.5% of macrophages infected for 4 h with *S. Typhimurium* (Sun *et al.*, 2007), less than 1% of cells were positive 16 h after infection with *B. abortus*. Therefore, although the intrinsic ability of *Brucella* and *Salmonella* flagellin to induce IL-1 β secretion from BMDM appeared to be similar (Fig. 6), *Brucella* might evade activation of a robust innate immune response from cytosolic PRRs by controlling the production and/or delivery of flagellin into the host cell. Accordingly, we could show that the attenuation of the *BruFliC^{ON}* strain that ectopically produces flagellin is due at least in part to a strong NLRC4 inflammasome activation *in vivo* (Fig. 7). Thus, we propose that the tight regulation of flagellin synthesis and/or delivery during infection is part of its stealthy strategy. This has also been suggested for *S. Typhimurium*, which downregulates the expression of *fliC* during macrophage infection (Cummings *et al.*, 2006).

In conclusion, we propose that flagellin is an important molecular actor of the interplay between *Brucella* and its host. Although flagellin escapes detection by TLR5 and *Brucella* controls its production and/or delivery to the infected host cell cytosol, its detection by cytosolic PRRs initiates a response that results in an immunological stand-off between *Brucella* and its host, leading to a persistent infection with limited inflammatory pathology. The increased bacterial tissue loads and destructive pathology, seen with the flagellin-deficient mutant, demonstrate that innate and possibly also adaptive recognition of flagellin is a process that is important to the chronic and stealthy nature of *Brucella* infection. As such, flagellin

could be considered as a 'host protective factor' (Shames and Finlay, 2010) in the context of brucellosis.

Experimental procedures

Bacteria and growth conditions

Bacterial strains and plasmids are listed in Table 1. Cultures of *Brucella* strains were freshly inoculated from frozen stock onto 2YT medium (10% yeast extract, 10 g l⁻¹ tryptone, 5 g l⁻¹ NaCl) plates before subculturing aerobically at 37°C in 2YT broth supplemented with appropriate antibiotics. LB broth was used for *Escherichia coli* and *Salmonella enterica* serotype Typhimurium (*S. Typhimurium*) cultures. Antibiotics were used at the following concentrations: carbenicillin, 100 mg l⁻¹; chloramphenicol, 30 mg l⁻¹; kanamycin, 60 mg l⁻¹; or nalidixic acid, 50 mg l⁻¹.

Molecular techniques

DNA manipulations were performed according to standard techniques (Ausubel *et al.*, 1991). Primers used are listed in Table 2.

Generation of the complementation vector pRH001-*fliC*. *fliC* coding sequence (cds) and its predicted upstream and downstream regulatory sequences were amplified by PCR using the *PfliC* and *tfliC* primers pair. The PCR product (*PfliC-fliC-tfliC*) was then cloned into the *EcorRV* site of pGEM. In a second step, this fragment was excised using BamHI and XbaI, and inserted into the corresponding sites of pMR10cat (R. Roberts, unpublished) in the opposite orientation to the *Plac*.

Generation of the *B. melitensis* 16M *FliC^{ON}* strain. The *fliC* over-expression vector pBBR1-*fliC* was obtained as follows: first, the constitutive promoter of the lac operon *Plac* was amplified by PCR using the *Plac* and *fliC-Plac* primers pair. In the resulting PCR product, *Plac* is flanked by translation stop codons in all three reading frame in 5' and by the 21st *fliC* coding sequence (cds) base pairs in 3'. *fliC* cds was amplified by PCR using the *BmfliC-F* and *BmfliC-R* primers. A third PCR using the *Plac* and *BmfliC-R* primers was used to ligate the two PCR products by cohesive ends. Stop codons and close fusion of *fliC* cds to *Plac* without any linker ensure the production of FliC flagellin that does not bear additional N-terminal amino acid residues. The PCR product (*XbaI-Plac-fliC-BamHI*) was then cloned into the *EcorRV* site of pGEM. In a last step, this fragment was excised using XbaI and BamHI, and inserted into the corresponding sites of pBBR1 MCS-I (Kovach *et al.*, 1994) in the opposite orientation to the endogenous *Plac*. This gave rise to pBBR1-*Plac-fliC*. This final construction was transformed into *E. coli* strain S17-1 (Simon *et al.*, 1983), and introduced into *B. melitensis* 16M by conjugation.

Generation of C-terminally FLAG-tagged flagellins. A derivative of the broad host range plasmid pBBR1MCS (pBBR1-FLAG) was first generated by ligating a fragment containing 'SphI-promoter-NdeI-SalI-3x-Flag-STOP-PstI-SacI' into pBBR1MCS4 treated with SphI and SacI. The *S. Typhimurium fliC* gene was amplified using primers StFliC-F and StFliC-R, and the resulting amplicon was ligated into NdeI and SalI-digested pBBR1-FLAG to yield plasmid pYHS1116, encoding StFliC-FLAG. The *B. abortus fliC*

Table 1. Bacterial strains and plasmids used in this study.

Designation	Genotype and/or phenotype	Source or reference
Strains		
<i>Brucella melitensis</i> strains		
16M	Wild type isolate	
$\Delta fliC$	$\Delta fliC::Kan$	Ferooz <i>et al.</i> (2011)
$\Delta flbT$	$\Delta flbT::Kan$	Ferooz <i>et al.</i> (2011)
$\Delta fliF$	$\Delta fliF::Kan$	Ferooz <i>et al.</i> (2011)
<i>BruFliC</i> ^{ON}	pBBR1- <i>fliC</i>	This work
<i>Brucella abortus</i> strain		
2308	Wild type isolate	
<i>Salmonella enterica</i> serovar Typhimurium strains		
14028	ATCC 14028 wild type	ATCC
IR715	14028 spontaneous Nal ^r	Stojiljkovic <i>et al.</i> (1995)
LT2	LT2 wild type	Lilleengen (1948)
EHW26	IR715 <i>fliC::Tn10 fljB::MudJ (fliCfljB)</i>	Raffatellu <i>et al.</i> (2005)
<i>Escherichia coli</i> strains		
CC118 λ pir	<i>araD139</i> $\Delta(ara, leu)$ 7697 $\Delta lacX74$ <i>phoA</i> Δ 20 <i>galE galk thi rpsE rpoB argE_{am} recA1</i> λ pir	Simon <i>et al.</i> (1983)
DH10B	F- <i>mcrA</i> $\Delta(mrr-hsdRMS-mcrBC)$ ϕ 80 <i>lacZ</i> Δ M15 $\Delta lacX74$ <i>recA1 araD139</i> $\Delta(ara, leu)$ 7697 <i>galU galk rpsL(Str^r) endA1 nupG</i>	Invitrogen
DH5 α	F- <i>mcrA</i> $\Delta(mrr-hsdRMS-mcrBC)$ ϕ 80 <i>lacZ</i> Δ M15 $\Delta(lacZYA-argF)$ U169 <i>deoR recA1 endA1 phoA supE44</i> λ - <i>thi-1 gyrA96 relA1</i>	Woodcock <i>et al.</i> (1989)
S17-1 λ pir	<i>recA thi pro r_K⁻ m_K⁺ RP4:2-Tc:MuKm Tn7</i> λ pir	Simon <i>et al.</i> (1983)
Plasmids		
pCR2.1	TOPO cloning vector	Invitrogen
pUC-K1XX	pUC4::Tn5 Kan ^r	Beck <i>et al.</i> (1982)
pBBR1MCS	<i>mob RK2, lacZα, Cm^r</i>	Kovach <i>et al.</i> (1994)
pRH001 <i>fliC</i>	pMR10 (Cm ^r , <i>B. melitensis</i> 16M <i>PfliC-fliC-tfiiC</i>)	This work
pBBR1- <i>fliC</i>	pBBR1MCS(Cm ^r , <i>B. melitensis</i> 16M <i>fliC</i>)	This work
pBBRFlag	pBBR1MCS::3xFLAG	This work
pYHS1116	pBBRFlag::StFliC	This work
pYHS1073	pBBRFlag::BaFliC	This work
pWSK29	Carb ^r , pSC101 <i>ori</i>	Wang and Kushner (1991)

gene was amplified using primers BaFliC-F and BaFliC-R and cloned in the same way to generate pYHS1073, encoding *BruFliC*-FLAG. In both constructs, expression of the recombinant proteins was controlled by a previously described constitutive *Brucella* promoter, BMEI0193 (Eskra *et al.*, 2001). The constructs were confirmed by DNA sequencing across the junction fragments. Plasmids pYHS1116 (*StFliC*-FLAG) and pYHS1073 (*BruFliC*-FLAG) were introduced into a *Salmonella fliC fljB* mutant (EHW26, Raffatellu *et al.*, 2005) by electroporation. The *B. abortus* and *B. melitensis* FliC proteins are identical except for a substitution of Ala156 to Thr in *B. abortus*.

Generation of fusions to TEM-1 β -lactamase. To express *BruFliC* fused with TEM-1, *B. abortus fliC* was amplified by using the primer pair BaFliC-F and BaFliC-R. The amplicon was cloned into pCR2.1, then subsequently digested with NdeI and PstI, and ligated pFlagTEM-1 (Raffatellu *et al.*, 2005) digested with the same enzymes to yield pBaFliC-TEM-1. The expression of *BruFliC::TEM-1* in pBaFliC-TEM-1 is under the control of inducible Trc promoter. Constructs expressing *StFliC::TEM-1* were described previously (Sun *et al.*, 2007).

Generation of GST-flagellin fusion proteins. For construction of plasmids expressing GST fused at the N-terminus of flagellins, flagellin genes were amplified to delete predicted N-terminal secretion domains. The *fliC* gene from *S. Typhimurium* was

amplified without its first 332 nucleotides using primer pair of *StFliC*-F2 and *StFliC*-STOP-R. Similarly, *B. abortus fliC* lacking its first 87 nucleotides was amplified using primer pair of BaFliC-F2 and BaFliC-R2. Both amplicons were cloned in pCR2.1, excised as BamHI/SalI fragments and ligated to BamHI/SalI-digested pGEX-4T-1. The cloning junctions were confirmed by DNA sequence analysis, and the resulting constructs, pGEX-*StFliC* and pGEX-BaFliC, were transformed into *E. coli* BL-21. Expression of GST::*StFliC* and GST::*BruFliC* was induced by IPTG, and the recombinant flagellins were purified using Glutathione-Sepharose 4B (GE Healthcare). Protein concentration was measured with DC protein assay (Bio-Rad).

Preparation of concentrated S. Typhimurium culture supernatant containing recombinant flagellins

S. Typhimurium strains were grown for 4 to 5 h at 37°C with vigorous shaking by diluting an overnight culture 1 to 100 in 20 ml LB broth plus 1 mM IPTG. Once the OD₆₀₀ reached 0.8 to 1.2, bacteria were removed by centrifugation at 4000 r.p.m. for 15 min and 12 ml of the resulting supernatant was passed through a 0.45 μ m filter and subject to concentration by using an Amicon Ultra-15 with cut-off of 5K (Millipore) followed by a wash with 10 ml of phosphate-buffered saline (PBS). Protein concentration was determined by DC protein assay (Bio-Rad) and SDS-PAGE

Table 2. Primers used in this work.

Primer	Sequence	Restriction site	Application
BaFliC-F	ACC <u>ATATGGCTAGCATTCTTACAAACTCGT</u> CG	NdeI	FLAG-tagged BaFliC and BaFliC::FT fusion protein
BaFliC-R	ACTGCAGTTAGCCGGGAACAGCGACAGGATCGAC	Sall	FLAG-tagged BaFliC and BaFliC::FT fusion protein
StFliC-F	ACC <u>ATATGGCACAAGTCAATTAACAACAGC</u>	NdeI	FLAG-tagged StFliC and StFliC::FT fusion protein
StFliC-R	AGTCGACTTAACCGCAGTAAAGAGGACGTTTTGC	Sall	FLAG-tagged StFliC and StFliC::FT fusion protein
StFliC-F2	GAATTCATGGCACAAAGTCAATTAACAACAGC	EcoRI	GST-StFliC fusion protein
StFliC-STOP-R	ACTCGAGTTAACGCAGTAAAGAGGACGTTTTGC	XhoI	GST fusion proteins
BaFliC-F2	GAATTCATGGCTAGCATTCTTACAAACTCG	EcoRI	GST fusion proteins
BaFliC-R2	ACTCGAGTTAGCCGGGAACAGCGACAG	XhoI	GST fusion proteins
Pflic	CGGGATCCAATGCCGGGATCATGTTGATGC	BamHI	Complementation plasmid
fliC	GC TCTAGATGCCAGACAGGATGTCGGGC	XbaI	fliC overexpression plasmid
Plac	GC TctagAtagAGCGCAACGCAATTAATGTGAG		
fliC-Plac	GTTTTGTAAGAATGCTAGCCATAGCTGTTCTCTGTGTGAAATTG		
BmfliC-F	ATGGCTAGCATTCTTACAAACTCGT		
BmfliC-R	CGGGATCCCTTAGCCGGGAACAGCG	BamHI	

Bold/Underlined: multiple cloning site; **Bold/Underlined/italicized:** restriction site utilized in cloning; Lower case: start or stop codon.

followed by Coomassie blue stain. The final protein concentration was adjusted to 1 mg μl^{-1} .

Generation of rabbit anti-BaFliC serum and Western blot

Brucella abortus fliC (BaFliC) was amplified using primers BaFliC-F and BaFliC-R and cloned into pET103 in frame with a 6xHis tag. The resulting BaFliC::6xHis fusion protein was produced and purified by using Ni-NTA kit (Qiagen). Rabbit serum against BaFliC was generated by Antagene (Antagene, CA, USA). For detection of secreted BaFliC the supernatant from 1 ml of culture was precipitated using trichloroacetic acid (TCA) and separated on a 12% SDS-PAGE gel. Proteins were electrotransferred to a polyvinylidene difluoride (PVDF) membrane. BaFliC was detected by using rabbit anti-BaFliC as primary antibody and as goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) as secondary antibody. *S. Typhimurium* Phase I flagellin (FliC) was detected using *Salmonella* Hi antiserum (Difco). C-terminal FLAG-tagged *S. Typhimurium* and *B. abortus* flagellins were detected using anti-FLAG monoclonal antibody (1:5000, Sigma) and a goat anti-mouse IgG antibody conjugated to HRP. HRP activity was detected with a chemiluminescent substrate (PerkinElmer Life Sciences). Flagellin produced by *B. melitensis* 16M was detected as described previously (Fretin *et al.*, 2005).

Measurement of TLR5 agonist activity of flagellins

The human colonic epithelial cell line T84 was cultured in Dulbecco's modified Eagle's medium (DMEM)-F12 medium (Gibco), containing 1.2 g l^{-1} sodium bicarbonate, 2.5 mM L-glutamine, 15 mM Hepes and 0.5 mM sodium pyruvate (Gibco), supplemented with 10% fetal calf serum (FCS). The day before assay, cells from 1/3 of an 80–90% confluent T75 flask were seeded per each 24-well plate containing DMEM-F12 and 2% FCS. HEK293 cells were cultured as previously described (Keestra *et al.*, 2010).

T84 cells in 24-well plates were either infected with 10 μl of bacteria grown as above or treated by adding 30 μl of concentrated bacterial culture supernatant and incubated for 4 h at 37°C under 5% CO_2 . For the HEK293 stably transfected with human TLR5, cells were grown in 48-well tissue culture plates and infected for 4–48 h with 10 μl of bacteria grown as described above or treated by adding 10 μl of concentrated bacteria culture supernatant and incubated for 8 h at 37°C under 5% CO_2 . Supernatants were aspirated and centrifuged for 10 min at 6000 r.p.m. to remove residual bacteria and cell debris before measurement of IL-8 concentration by ELISA.

MAPK phosphorylation assay

T84 cells were seeded in six-well plates at a density of 4×10^8 cells per well and incubated for 24 h in DMEM/F12 + 10% fetal bovine serum. The following day, cells were rinsed with PBS and the medium replaced with serum-free medium. For analysis of MAP kinase phosphorylation, cells were treated with concentrations of GST-*Bru*FliC or GST-StFliC ranging from 250 ng ml^{-1} to 1 $\mu\text{g ml}^{-1}$. As a negative control, cells were treated with the highest concentration of flagellin (1 $\mu\text{g ml}^{-1}$) that had previously been treated with proteinase K (20 mg ml^{-1} proteinase K for 1 h

at 37°C, then for 10 min at 75°C to inactivate the protease). After 30 and 90 min, cells were lysed 0.1 ml in phosphosafe extraction reagent (Novagen) containing 2.5% protease inhibitor (Sigma) according to the instructions of the manufacturer. The protein concentration was determined using the Micro BCA kit (Pierce). Total protein (0.01 mg) was resolved by SDS-PAGE and transferred to a polyvinylidene fluoride membrane. Primary antibodies were purchased from Cell Signalling Technology, including the following phosphorylation-specific antibodies: p-ERK and p-p38 (Thr180/Tyr182). Secondary antibodies (goat anti-rabbit conjugated to HRP) were purchased from Jackson Immunoresearch and used according to the recommendations of the manufacturer. Peroxidase activity was visualized using Immobilon Western Chemiluminescent HRP Substrate (Millipore). For each primary antibody, a separate membrane was used.

Detection of flagellin in the cytosol of infected macrophages

The β -lactamase translocation assay was performed as previously described (Sun *et al.*, 2007). Briefly, J774A.1 mouse macrophages were seeded in 96-well coverglass bottom plates and infected with *B. abortus* 2308 expressing either a *BruFliC::Flag-TEM-1* fusion protein, or an irrelevant control (Glutathione-S-transferase::Flag-TEM-1) at a multiplicity of infection of 500. Plates were centrifuged at 250 *g* for 5 min at room temperature to synchronize infection. After incubation for 1 h at 37°C in 5% CO₂, free bacteria were removed from the cells by three washes with PBS. A volume of 0.2 ml of DMEM supplemented with 10% heat-inactivated fetal bovine serum, 1% non-essential amino acids, 1 mM glutamine containing 1 mM IPTG and 100 μ g ml⁻¹ gentamicin was added to each well, and plates were incubated at 37°C in 5% CO₂. After 16 h, cells were washed once with Hank's balanced salt solution (Invitrogen) and loaded with the fluorescent substrate CCF2/AM (1 mM, Invitrogen) for 1.5 h at room temperature using the standard loading protocol recommended by the manufacturer. Fluorescence microscopy analysis was performed using an Axiovert M200 (Carl Zeiss), equipped with a CCF2 filter set (Chroma Technology). Fluorescence micrographs were captured using a Zeiss Axiocam MRC5 and Zeiss AxioVision 4.5 software. Images were imported into Adobe PhotoShop for colour adjustment. The number of blue cells containing cleaved CCF2/AM was counted visually and expressed as the percentage of total cells in the well. The experiment was performed four times and the result expressed as geometric mean and range of the four experiments.

Bone marrow-derived macrophages

Bone marrow-derived macrophages were isolated from C57BL/6, or congenic mutant mice following standard protocols as described previously (Sun *et al.*, 2007).

Macrophage infection

For assaying inflammasome activation, 24-well microtitre plates were seeded with BMDM at a concentration of 2×10^5 cells/well in 0.5 ml of RPMI-sup and incubated overnight at 37°C in 5% CO₂. For priming of macrophages, cells were treated for 4 h before

infection with LPS (100 ng ml⁻¹), as previously described (Franchi *et al.*, 2006).

Inocula of *B. melitensis* 16M were prepared by growing with shaking in TSB for 24 h. Bacteria were treated with a non-agglutinating (1:4000) dilution of anti-*Brucella* rabbit serum (Difco) for 1 h at 37°C, as described (Rolan and Tsolis, 2007), then diluted in RPMI-sup to a concentration of 4×10^7 CFU ml⁻¹. Approximately 2×10^7 bacteria in 0.5 ml of RPMI-sup, containing *B. melitensis* 16M wt or its isogenic *fliC* mutant, were added to each well of macrophages. Three independent assays were performed with triplicate samples, and each experiment included control (C57BL/6) macrophages together with macrophages from mutant mice. Microtitre plates were centrifuged at 250 *g* for 5 min at room temperature in order to synchronize infection. Cells were incubated for 20 min at 37°C in 5% CO₂, and free bacteria were removed by three washes with PBS. RPMI-sup plus 50 mg ml⁻¹ gentamicin was added to the wells, and the cells were incubated at 37°C in 5% CO₂. After 1 h, the RPMI-sup plus 50 μ g ml⁻¹ gentamicin was replaced with medium containing 25 μ g ml⁻¹ gentamicin. Wells were sampled after infection by aspirating the medium, lysing the macrophages with 0.5 ml of 0.5% Tween-20 and rinsing each well with 0.5 ml of PBS. Viable bacteria were quantified by dilution in sterile PBS and plating on TSA containing appropriate antibiotics.

Liposome-mediated delivery of flagellins to the macrophage cytosol

Recombinant flagellin proteins were delivered to the macrophage cytosol using the cationic lipid DOTAP (Roche), as described previously (Franchi *et al.*, 2006). Briefly, 50 ml of DOTAP was incubated for 30 min in serum-free media with 2 mg of recombinant flagellins purified as described above. After incubation, 3.5 ml of serum-free media was added and 500 μ l was used to stimulate 1×10^6 macrophages seeded in 24-well microtitre plates for 3 h.

Measurement of cytokines

Mouse IL-1 β was measured in culture supernatants by enzyme-linked immunosorbent assay (ELISA) (R&D Systems). Human IL-8 was detected using an ELISA kit from BioLegend.

Mice

Wild type (wt) BALB/c, wt C57BL/6, C57BL/6 *Nlr4*^{-/-} (obtained from Dr V.M. Dixit and described in Mariathasan *et al.*, 2004) and C57BL/6 *Casp1*^{-/-} (obtained from Dr R. Flavell and described in Kuida *et al.*, 1995) mice were used in this study. They were bred in the animal facility of the University of Namur (Belgium). The animal handling and procedures of this study were in accordance with the current European legislation (directive 86/609/EEC) and in agreement with the corresponding Belgian law 'Arrêté royal relatif à la protection des animaux d'expérience du 6 avril 2010 publié le 14 mai 2010'. The complete protocol was reviewed and approved by the Animal Welfare Committee of the University of Namur, Belgium (Permit Number: 05-558).

Infection of mice

Mice were injected intraperitoneally (i.p.) with 4×10^4 CFUs of *B. melitensis* 16M in 500 μ l of PBS. Control animals were

injected with the same volume of PBS. Infectious doses were validated by plating serial dilutions of the inocula. At selected time intervals, mice were sacrificed by cervical dislocation. Immediately after being killed, spleen and liver were collected for bacterial counts and histopathological analyses. For bacterial counts, spleens and livers were homogenized in PBS/0.1% X-100 triton (Sigma). Serial dilutions were plated on 2YT media plates for enumeration of tissue-associated CFU.

Histology

Spleens were fixed for 24 h in Bouin's fixative, dehydrated for 24 h in methanol, then incubated in toluol and finally in warm paraffin prior to paraffin embedding. Sections (5 µm) were rehydrated and stained with hemalun, erythrosin and safran. Blinded histopathology scoring for splenic granuloma formation was performed by a pathologist (M.N.X.), according to the following criteria. 0, < 5% of splenic parenchyma containing granulomas; 1, 5–20%; 2, 20–40%; 3, 40–60%; 4, > 60%.

Immunofluorescence microscopy

Spleens were fixed for 6 h at 4°C in 2% paraformaldehyde (pH 7.4), washed in PBS, incubated overnight at 4°C in a 20% PBS-sucrose solution under agitation, and washed again in PBS. Tissues were embedded in the Tissue-Tek OCT compound (Sakura), frozen in liquid nitrogen, and cryostat sections (10 µm) were prepared. Tissues sections were rehydrated in PBS, then incubated successively in a PBS solution containing 1% blocking reagent (Boehringer) (PBS-BR 1%) and in PBS-BR 1% containing any of the following mAbs or reagents: DAPI nucleic acid stain, Alexa Fluor 350 phalloidin, M1/70 (anti-CD11b, BD Biosciences), home-made anti-*B. melitensis* 16M serum (Copin *et al.*, 2012). Slides were mounted in Fluoro-Gel medium (Electron Microscopy Sciences, Hatfield, PA, USA). Labelled tissue sections were visualized under a Zeiss fluorescent inverted microscope (Axiovert 200) equipped with high-resolution monochrome camera (AxioCam HR, Zeiss).

Statistical analysis

ANOVA I was used for infection data analysis after testing the homogeneity of variance (Bartlett test). Average comparisons were performed by pairwise Scheffe's test. A Mann-Whitney test was used for analysis of histopathology scoring. Error bars represent standard deviation.

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