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

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Running title: *Brucella* flagellin as innate immune signal

Abstract

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, and that the cytosolic adaptor ASC was involved in its recognition. Accordingly, we showed that FliC was translocated into the cytosol of infected cells. However, our work also suggested that the lack of TLR5 activity of *Brucella* flagellin and the tight 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, since 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 during infection plays a crucial role in the immunologic standoff 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 *et al.*, 2011), and Nod-like receptors (NLRs), which are cytosolic receptors responding to PAMPs and endogenous danger signals (Lamkanfi *et al.*, 2009, Brodsky *et al.*, 2009a). 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 *et al.*, 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 *et al.*, 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-κB, and the activation of mitogen activated protein kinases (MAPK), ultimately inducing the secretion of proinflammatory cytokines and chemokines, such as IL-8 (Gewirtz *et al.*, 2001, Eaves-Pyles *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 *et al.*, 2011, Zhao *et al.*, 2011). Activation of caspase-1 within the NLRC4 inflammasome leads to the maturation and release of biologically active proinflammatory cytokines IL-1ß 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), *Legionella pneumophila* (Hawn *et al.*, 2003) and *Pseudomonas aeruginosa* (Feuillet *et al.*, 2006, 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 *B. 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 *et al.*, 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, since 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* (Δ*fliC*) and *flbT* (Δ*flbT*) (Ferooz *et al.*, 2011) were used to infect murine macrophages and BALB/c mice. The FlbT 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 Δ*fliC* nor in the Δ*flbT* strain harvested at the early exponential phase of growth, whereas the protein is produced by the isogenic wt strain (Fig. 1A).

We first compared the intracellular growth of *B. melitensis* Δ*fliC* and Δ*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 not shown). Consistent with a normal multiplication in endoplasmic reticulum-derived vacuoles, both Δ*fliC* mutant and its isogenic parental strain were found to replicate within calnexin-positive compartments of HeLa cells at 24h 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 Δ*fliC*, Δ*flbT* and Δ*fliF* non-polar mutants. None of the mutants was significantly attenuated 5 days p.i., as compared with the parental strain (Fig. 1C). Moreover, we could confirm that the basal body protein FliF is required for full virulence. Indeed, the Δ*fliF* mutant was attenuated at 3 and 4 weeks p.i. (Fig. 1C). In contrast, the virulence of the Δ*fliC* strain was exacerbated when compared to its isogenic parental strain, as Δ*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 Δ*fliC* mutant (data not shown). Similarly, an enhanced persistence of the Δ*fliC* strain in the spleens of the resistant C57BL/6 mice has also been observed (data not shown). 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 Δ*fliC* strain (Fig. 1A), allowed partial complementation of the phenotype of the newly constructed Δ*fliC* mutant at 28 days p.i. and full complementation at 60 days p.i. (Fig. 1C). Moreover, we could show that the Δ*flbT* mutant had similar infection kinetics than the Δ*fliC* strain in the spleen of BALB/c mice (Fig. 1C). 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* Δ*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 gr (4-fold 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 5 or 6 times the

normal spleen weight by the end of the experiment (Fig. 2A for Δ*fliC*, data not shown for Δ*flbT*). A similar exacerbation of splenomegaly was also observed in C57BL/6 mice at 21 days p.i with the Δ*fliC* mutant (data not shown). This was in accordance with the enhanced persistence of the flagellin-deficient mutants in mice (Fig. 1C).

We further examined the splenic histopathology of BALB/c mice infected for 28 days with wt or Δ*fliC B. melitensis* strain. At this time, mice infected with the Δ*fliC* strain showed a markedly exacerbated splenic inflammation characterized by increased vasodilation, thrombosis, neutrophil infiltration and granuloma formation (Fig 2B and 2C). 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 Δ*fliC* or Δ*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 *Bru*FliC^{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, *Bru*FliC^{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 *Bru*FliC^{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 post infection of BALB/c mice, 0.5 to 1 log fewer CFU of *Bru*FliC^{ON} bacteria were recovered at 12, 21 and 28 days p.i. (Fig.

3C). Reduced colonization of *Bru*FliC^{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 Δ*fliC* and *Bru*FliC^{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* (*Bru*FliC-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 *Bru*FliC-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 motility, since strains expressing either StFliC-FLAG or *Bru*FliC-FLAG were aflagellate and non-motile (data not shown).

Culture supernatants of *S.* Typhimurium *fliCfljB* expressing recombinant flagellins were used to treat two TLR5-expressing cell lines: the colonic epithelial cell line T84 and HEK293/hTLR5 (Fig. 4B and 4C). 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, since culture supernatants from the *fliCfljB* mutant elicited little (Fig. 4C) or no (Fig. 4B) IL-8. In contrast to StFliC-FLAG, expression of *Bru*FliC-FLAG did not elicit IL-8 secretion above the level of the *fliCfljB*

mutant. Similar results were obtained when T84 or HEK-293/hTLR5 cells were infected with *S.* Typhimurium strains expressing recombinant flagellins (data not shown). The response to *Bru*FliC did not appear to be delayed, since extending the time of the assay to 24h did not allow detection of a response comparable to that elicited by StFliC-FLAG. As a second readout for TLR5 signaling, we assayed activation of mitogen-activated protein kinases (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-*Bru*FliC, and notably no increase in phosphorylation of ERK could be detected after treatment with GST-*Bru*FliC (Fig. 4D). Taken together, these results demonstrate that compared to *S.* Typhimurium flagellin, the ability of *Brucella* flagellin to stimulate TLR5 signaling 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 *et al.*, 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 (pFLAG-TEM1; 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 4 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-1β secretion from primary bone marrow-derived macrophages (BMDM). Compared to *B. melitensis* wt, the Δ*fliC* mutant elicited significantly reduced IL-1β secretion (Fig. 5A). This reduction was not the result of differing numbers of intracellular bacteria of the Δ*fliC* mutant, since both the Δ*fliC* mutant and wt *B. melitensis* were present in the same numbers (data not shown). This partial reduction in IL-1β 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). The *Bru*FliC^{ON} strain elicited significantly more IL-1β secretion than the control strain, 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-1β, NLRC4 was not required for flagellin-dependent stimulation of IL-1β secretion by BMDM *in vitro*.

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

Since *B. melitensis* is known to inhibit innate immune signalling (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-*Bru*FliC and GST-StFliC were introduced into the cytosol of BMDM using the cationic lipid DOTAP (Franchi *et al.*, 2006). Both

*Bru*FliC and StFliC elicited dose-dependent secretion of IL-1β 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-1β (Fig. 6A and data not shown). Comparison of IL-1β secreted in response to equal amounts of StFliC or *Bru*FliC suggested that the proinflammatory activity of StFliC was slightly higher than that of *Bru*FliC (Fig. 6A). Secretion of IL-1β 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, *Bru*FliC elicited IL-1β secretion that required ASC, but was independent of NLRC4, at least in cultured BMDM (Fig. 6B and Fig. 6C). 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 *et al.*, 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* strains (Fig. 1C 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 *Bru*FliC^{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 *Bru*FliC^{ON}-

infected C57BL/6 mice contained less CFUs than these 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* (Fig. 6), *Brucella* flagellin can activate the NLRC4 inflammasome *in vivo*. Nevertheless, 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 Δ*fliC* mutant (Fig. 7). This suggests that both inflammasome-dependent and inflammasome-independent control of infection operates downstream detection of *Brucella* flagellin *in vivo*.

B. melitensis Δ*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 Δ*fliC* strain. 5 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 cells clusters counted in splenic sections of Δ*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 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* (Δ*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), *Legionella 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 insights 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 *et al.*, 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 Δ*fliC* or Δ*flbT* mutants cannot by itself account for the inability of the host to control infection. Indeed, the relative difference of virulence between *B. melitensis* 16M wt and Δ*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*. Actually, the observation that the *Bru*FliC^{ON} strain is still attenuated (a reproducible 0.5 log decreased CFUs in the spleen) compared to *B. melitensis* 16M wt in *Nlrc4*$^{-/-}$ and *Casp1*$^{-/-}$ mice 21 days p.i. 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. This would be similar to what has been described for *L. pneumophila* that triggers an ASC-dependent activation of caspase-1 in macrophages, in addition to the NLRC4-dependent activation triggered by cytosolic flagellin (Case *et al.*, 2009). Activation of these innate immune pathways by flagellin would play a role in limiting replication of *Brucella in vivo*. However, the immune effector mechanisms involved remain to be uncovered. Processing of the proinflammatory cytokines pro-IL-1ß 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 *et al.*, 2009b).

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 *et al.*, 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 Δ*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 Δ*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 *et al.*, 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 *M. 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* (Ren *et al.*, 2006, Molofsky *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, since 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 TEM1 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 4h with *S.* Typhimurium (Sun *et al.*, 2007), less than 1% of cells were positive 16h after infection with *B. abortus*. Therefore, although the intrinsic ability of *Brucella* and *Salmonella* flagellin to induce IL-1ß 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 *Bru*FliC^{ON} strain that ectopically produces flagellin is due 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 immunologic standoff 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 demonstrates 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 *et al.*, 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 liter^{-1} tryptone, 5 g liter^{-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/liter; chloramphenicol, 30 mg/liter; kanamycin, 60 mg/liter; or nalidixic acid, 50 mg/liter.

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 P*fliC* and t*fliC* primers pair. The PCR product (P*fliC-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 pMR10*cat* (R. Roberts, unpublished) in the opposite orientation to the P*lac*.

Generation of the B. melitensis 16M FliC^{ON} strain: The *fliC* overexpression vector pBBR1-*fliC* was obtained as follows: first, the constitutive promoter of the lac operon P*lac* 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 Bm*fliC*-F and Bm*fliC*-R primers. A third PCR using the Plac and Bm*fliC*-R primers was used to ligate the two PCR products by cohesive ends. Stop codons and close fusion of *fliC* cds to P*lac* without any linker ensure the production of FliC flagellin that does not bear additional N-terminal amino acid residues. The PCR product (XbaI-P*lac-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 P*lac*. This gave rise to pBBR1-P*lac-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 "*Sph*I-promoter-*Nde*I-*Sal*I-3x-Flag-STOP-*Pst*I-*Sac*I" into pBBR1MCS4 treated with *Sph*I and *Sac*I. The *S.* Typhimurium *fliC* gene was amplified using primers StFliC-F and StFliC-R, and the resulting amplicon was ligated into *Nde*I and *Sal*I-digested pBBR1-FLAG to yield plasmid pYHS1116, encoding StFliC-FLAG. The *B. abortus fliC* gene was amplified using primers BaFliC-F and BaFliC-R and cloned in the same way to generate pYHS1073, encoding BaFliC-FLAG. In both constructs, expression of the recombinant proteins was controlled by a previously described constitutive *Brucella* promoter, BMEII0193 (Eskra *et al.*, 2001). The constructs were confirmed by DNA sequencing across the junction fragments. Plasmids pYHS1116 (StFliC-FLAG) and pYHS1073 (BaFliC-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 BaFliC fused with TEM1, *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 *Nde*I and *Pst*I, and ligated pFlagTEM1 (Raffatellu *et al.*, 2005) digested with the same enzymes to yield pBaFliCTEM1. The expression of BaFliC::TEM1 in pBaFliCTEM1 is under the control of inducible Trc promoter. Constructs expressing StFliC::TEM1 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 *Bam*HI/*Sal*I fragments, and ligated to *Bam*HI/*Sal*I –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::BaFliC was induced by IPTG, and the recombinant flagellins were purified using Glutathione-Sepharose 4B (GE Healthcare). Protein concentration was measured with DC protein assay (BioRad).

Construction of plasmids expressing native S. Typhimurium and B. abortus flagellins: The pSC101 *ori*-based low copy number plasmid pWSK29 (Genbank AF016889) was digested with PvuII and BsaAI to remove the *lac* promoter, *lacZ*□ fragment, and most of the f1 *ori*, as represented by a 4.7kb fragment product (Figure 2a). The 4.7kb plasmid fragment was gel purified then treated with Antarctic phosphatase (New England Biolabs, NEB). Primers 102 and 103 were used to PCR amplify the -134 to -6 region of *S.* Typhimurium LT2, with primer 103 adding an XbaI site which serves to replace the -5 to -1 region of *fliC* and overlaps the translation start site. Primer 103 also contains a multiple cloning site (MCS), adding unique restriction sites (in the context of PvuII/BsaAI-digested pWSK29) downstream of the XbaI site. Both primer 102 and 103 contain PvuII sites at their 5' ends, so the resulting PCR product was cleaved with PvuII, gel purified, and blunt ligated to the pWSK29 PvuII/BsaAI fragment with Quick T4 DNA Ligase (NEB) and heat shocked into *E. coli* DH5α. Clones were screened for the loss of an EcoRV site (proper PvuII site ligated), gain of a single BstBI site (one promoter region insertion) and the orientation with the *fliC* promoter facing away from the pSC101 *ori* (the same directionality as the *lac* promoter in pWSK29) was screened for by BglII/PstI double digestion. Clones fitting this description were sequenced using

primers 108 and 109, which flank the insertion site in pWSK29, by SeqWright (Houston, TX, USA). An accurate clone was designated pSPN30.

Preparation of concentrated *S.* Typhimurium culture supernatant containing recombinant flagellins

S. Typhimurium strains were grown for 4 to 5 hours 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 OD_{600} reached 0.8 to 1.2 bacteria were removed by centrifugation at 4000 rpm for 15 min and 12 ml of the resulting supernatant was passed through a 0.45 μm filter and subject to concentration by using an Amicon Ultra-15 with cutoff of 5K (Millipore) followed by a wash with 10 ml PBS. Protein concentration was determined by DC protein assay (BioRad) and SDS-PAGE followed by Coomassie blue stain. The final protein concentration was adjusted to 1□g/ul.

Generation of rabbit anti-BaFliC serum and Western blot

B. 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 Inc., Calif.). For detection of secreted BaFliC the supernatant from 1 ml 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 T-84 was cultured in were maintained in Dulbecco's modified Eagle medium (DMEM)-F12 medium (Gibco), containing 1.2 g/liter 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 a 80 to 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 hours 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 hours at 37°C under 5% CO_2. Supernatants were aspirated and centrifuged for 10 min at 6,000 rpm to remove residual bacteria and cell debris before measurement of IL-8 concentration by ELISA.

Mitogen-activated protein kinase (MAPK) phosphorylation assay

T84 cells were seeded in six well plates at a density of 4×10^8 cells per well and incubated for 24h in DMEM/F12 + 10% FBS. 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-BaFliC or GST-StFliC ranging from 250ng/ml

to 1 µg/ml. As a negative control, cells were treated with the highest concentration of flagellin (1µg/ml) that had previously been treated with proteinase K (20mg/ml proteinase K for 1h 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 horseradish peroxidase) 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 BaFliC::Flag-TEM-1 fusion proteins, 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 hour 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 Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 1% non-essential amino acids, 1 mM glutamine containing 1 mM IPTG and 100 ug/ml 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 (1mM, 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 color 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 microtiter plates were seeded with bone marrow-derived macrophages at a concentration of 2 x10^5 cells/well in 0.5 ml of RPMIsup and incubated over night at 37°C in 5% CO_2. For priming of macrophages, cells were treated for 4h before infection with LPS (100 ng/ml), as previously described (Franchi *et al.*, 2006). Inocula of *B. melitensis* 16M were prepared by growing with shaking in TSB for 24h. Bacteria were treated with a non-agglutinating (1:4,000) dilution of anti-*Brucella* rabbit serum (Difco) for 1h at 37 ^oC, as described (Rolan *et al.*, 2007) then diluted in RPMIsup to a concentration of 4 x 10^7 CFU/ml. Approximately 2 x 10^7 bacteria in 0.5 ml of RPMIsup, 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. Microtiter plates were centrifuged at 250 x g for 5 min at room temperature in order to synchronize infection. Cells were incubated for 20 min at 37°C in 5% CO_2, and free bacteria were removed by three washes with phosphate-buffered saline (PBS). RPMIsup plus 50mg gentamicin per ml was added to the wells, and the cells were incubated at 37°C in 5% CO_2. After 1 h, the RPMIsup plus 50µg/ml gentamicin was replaced with medium containing 25µg/ml 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 serum-free media was added and 500 □l was used to stimulate 1 x 10^6 macrophages seeded in 24-well microtiter plates for 3h.

Measurement of cytokines

Mouse IL-1ß was measured in culture supernatants by enzyme-linked immunoabsorbent 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 *Nlrc4*$^{-/-}$ (obtained from Dr. VM. 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 Facultés Universitaires Notre-Dame de la Paix (FUNDP, Belgium)(Permit Number: 05-558).

Infection of mice

Mice were injected intraperitoneally (i.p.) with 4 x 10^4 CFUs of *B. melitensis* 16M in 500µl 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 histopathologic 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 24h in Bouin's fixative, dehydrated for 24h in methanol, then incubated in toluol and finally in warm paraffin prior to paraffin embedding. Sections (5µm) were rehydrated and stained with hemalun, erythrosin and safran. Blinded histopathology scoring for splenic granuloma formation was performed by a pathologist (MX), according to

the following criteria. 0, <5% of splenic parenchyma containing granulomas; 1, 5-20%; 2, 20-40%; 3, 40-40%; 4, >60%.

Immunofluorescence microscopy

Spleens were fixed for 6h 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 (10µm) were prepared. Tissues sections were rehydrated in PBS, then incubated successively in a PBS solution containing 1% blocking reagent (Boeringer) (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), homemade anti-*B. melitensis* 16M serum (Copin *et al.*, 2012). Slides were mounted in Fluoro-Gel medium (Electron Microscopy Sciences, Hatfield, PA). Labelled tissues 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 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. Errors bars represent standard deviation.

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

Designation	Genotype and/or Phenotype	Source or Reference
Strains		
Brucella melitensis strains		
16M	wild type isolate	
Δ*fliC*	Δ*fliC*::Kan	(Ferooz *et al.*, 2011)
Δ*flbT*	Δ*flbT*::Kan	(Ferooz *et al.*, 2011)
Δ*fliF*	Δ*fliF*::Kan	(Ferooz *et al.*, 2011)
*Bru*FliC^{ON}	pBBR1-*fliC*	This work
Brucella abortus strain		
2308	wild type isolate	
Salmonella enterica serovar Typhimurium strains		
14028	ATCC 14028 Wild-Type	ATCC
IR715	14028 Spontaneous Nal^R	(Stojiljkovic *et al.*, 1995)
LT2	LT2 Wild-Type	(Lilleengen, 1948)
EHW26	IR715 *fliC*::Tn*10 fljB*::Mud*J* (*fliCfljB*)	(Raffatellu *et al.*, 2005)
Escherichia coli strains		
CC118 λpir	*araD139* Δ(*ara*, *leu*)7697 Δ*lacX74 phoA*Δ*20 galE galK thi rpsE rpoB argE$_{am}$ recA1* λ*pir*	(Simon *et al.*, 1983)
DH10B	F- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80*lacZΔM15 ΔlacX74 recA1 araD139* Δ(*ara, leu*)7697 *galU galK rpsL*(Str^R) *endA1 nupG*	Invitrogen
DH5□	F- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80*lac*Δ*Z*□*M15* Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 phoA supE44* λ*- thi-1 gyrA96 relA1*	(Woodcock *et al.*, 1989)
S17-1 λpir	*recA thi pro* r_K^- m_K^+ *RP4*:*2-Tc*:MuKm Tn*7* □*pir*	(Simon *et al.*, 1983)
Plasmids		
pCR2.1	TOPO cloning vector	Invitrogen
pUC-KIXX	pUC4::Tn*5* KanR	(Beck *et al.*, 1982)
pBBR1MCS	*mob* RK2, *lacZ*α, Cm^R	(Kovach *et al.*, 1994)
pRH001*fliC*	pMR10 (Cm^R, *B. melitensis* 16M P*fliC-fliC-tfliC*)	This work
pBBR1-*fliC*	pBBR1MCS(Cm^R, *B. melitensis* 16M *fliC*)	This work
pBBRFlag	pBBR1MCS::3xFLAG	This work
pYHS1116	pBBRFlag::StFliC	This work
pYHS1073	pBBRFlag::BaFliC	This work
pWSK29	Carb^R, pSC101 *ori*	(Wang *et al.*, 1991)

943

Table 2: Primers used in this work

Primer	Sequence	Restriction site	Application
BaFliC-F	**AC*CATATG***GCTAGCATTCTTACAAACTCGTCG	NdeI	FLAG-tagged BaFliC and BaFliC::FT fusion protein
BaFliC-R	**AC*TGCAG***TTAGCCGCGGAACAGCGACAGGAT CGAC	SalI	
StFliC-F	**AC*CATATG***GCACAAGTCATTAATACAAACAGC	NdeI	FLAG-tagged StFliC and StFliC::FT fusion protein
StFliC-R	**A*GTCGAC***TTAACGCAGTAAAGAGAGGACGTTT TGC	SalI	
StFliC-F2	***GAATTC***ATGGCACAAGTCATTAATACAAACAG C	EcoRI	GST-StFliC fusion protein
StFliC-STOP-R	***ACTCGA***GTTAACGCAGTAAAGAGAGGACGTTT TGC	XhoI	
BaFliC-F2	***GAATTC***ATGGCTAGCATTCTTACAAACTCG	EcoRI	GST fusion proteins
BaFliC-R2	***ACTCGAG***TTAGCCGCGGAACAGCGACAG	XhoI	GST fusion proteins
PfliC	**CG*GGATCC***AATGCCCGGGATCATGTTGATGC	BamHI	complementation plasmid
tfliC	**GC*TCTAGA***TGCCAGACAGGATGTCGGGC	XbaI	
Plac	**GC*TCtagA***tagAtagAGCGCAACGCAATTAATGT GAG	XbaI	fliC overexpression plasmid
fliC-Plac	GTTTGTAAGAATGCTAGCCATAGCTGTTTCCT GTGTGAAATTG		
BmfliC-F	ATGGCTAGCATTCTTACAAACTCGT		
BmfliC-R	**CG*GGATCC***TTAGCCGCGGAACAGCG	BamHI	

Bold: Extra 5' DNA; **Bold/Underlined**: Multiple cloning site; ***Bold/Underlined/Italicized***:

Restriction site utilized in cloning; Lower case: Start or stop codon.

*

Figure legends

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. Δ*fliC* p*fliC* is the complemented strain. **(B)** Intracellular replication of *B. melitensis* 16M wt and Δ*fliC* strains in RAW264.7 murine macrophages. Error bars represent the standard deviation of triplicates in one representative experiment out of three. **(C)** Infection kinetics in the spleens of wt BALB/c mice (n=5) inoculated intraperitoneally (i.p.) with 4 x 10^4 CFUs of *B. melitensis* 16M wt, Δ*fliC*, complemented Δ*fliC* p*fliC* **(D)** Infection kinetics in the spleens of wt BALB/c mice (n=5) inoculated intraperitoneally (i.p.) with 4 x 10^4 CFUs of *B. melitensis* 16M wt, Δ*flbT*, or Δ*fliF* strains. Data represent the mean CFUs per organ and error bars represent standard deviation. Results have been analyzed 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.

Fig. 2. Enhanced persistence of *B. melitensis* Δ*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 x 10^4 CFUs of wt or Δ*fliC* strains of *B. melitensis* 16M. Data represent the mean spleen weight and error bars represent standard deviation. Results have been analyzed 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 the Material and methods. Data were analysed using a Mann Whitney test, and the mean histopathology scores were significantly different (P=0.009) **(C)** Representative photomicrographs (x10) of histopathology of spleens from BALB/c mice uninfected or infected for 28 days with *B. melitensis* wt or Δ*fliC* strain. WP, white pulp; T, thrombosis; black arrows, granuloma; white arrowhead, neutrophil infiltration. These results are representative of at least two independent experiments.

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 *Bru*FliC^{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 *Bru*FliC^{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 x 10^4 CFUs of wt or *Bru*FliC^{ON} strain. Data represent the mean CFUs per organ and error bars represent standard deviation. Results have been analyzed 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.

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

(A-C) FLAG-tagged flagellins from *S. enterica* serotype Typhimurium (StFliC) or *Brucella abortus* (*Ba*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 BaFliC-FLAG (lane 4). Flagellins were detected both in the pellets (left panel) and in the concentrated supernatants (right panel) of *S.* Typhimurium strains. 30ng of concentrated supernatant proteins from *S.* Typhimurium strains expressing recombinant flagellins were used to treat HEK293/hTLR5 cells for 4 or 24h **(B)** and T84 cells for 8h **(C)**. IL-8 in cell supernatants was measured by ELISA. **(D)** Activation of p38 and ERK MAPK in T-84 cells by purified recombinant flagellins from *Brucella* (GST-BaFliC) and *S.* Typhimurium (GST-StFliC) 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 can enter the cytosol of infected macrophages and induces IL-1β in an NLRC4-independent manner. (A) Bone marrow-derived macrophages from C57BL/6 mice were inoculated with *B. melitensis* 16M wt or the Δ*fliC* mutant and IL-1β was measured in the culture supernatants by ELISA at 24h p.i. Results are shown as the mean ± standard deviation of data from five independent experiments. **(B)** Bone marrow-derived macrophages from C57BL/6 or *Nlrc4*$^{-/-}$ mice were inoculated with *B. melitensis* 16M wt or the *Bru*FliC^{ON}strain. IL-1β in the supernatant was measured at 6h after inoculation. Data shown are combined from three independent experiments with triplicate samples, and represent the mean ± standard deviation of all data.

Fig. 6. Introduction of recombinant *Brucella* flagellin into the host cell cytosol results in ASC-dependent, but NLRC4-independent secretion of IL-1β.

Graded amounts of GST-BaFliC and GST-StFliC fusion proteins were delivered to the cytosol of LPS-primed bone marrow-derived macrophages from C57BL/6 **(A)**, *Nlrc4*$^{-/-}$ **(B)** or *Asc*$^{-/-}$ **(C)** mice, using the cationic lipid DOTAP. Treated macrophages were incubated for 3h before measurement of IL-1β 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 x 10^4 CFUs of *B. melitensis* wt, *Bru*FliC^{ON} or Δ*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 respectively significant ($p<0.05$) and highly significant ($p<0.01$) differences in relation to C57BL/6 wt infection by wt bacteria.

Fig. 8. The distribution of *Bru*-positive cells is different in the spleen of mice infected by the Δ*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 Δ*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.

Acknowledgements

We thank V. Dixit, J. Tschopp and A. Tardivel for providing us with the NLRC4 KO mice. Part of this work has been granted by an ARC Convention from the French community of Belgium (N° 08/13-015). M. Terwagne holds a PhD grant from FNRS (Fond National pour la Recherche Scientifique) and J. Ferooz holds a PhD grant from FRIA (Fonds pour la formation à la Recherche dans l'Industrie et l'Agriculture).

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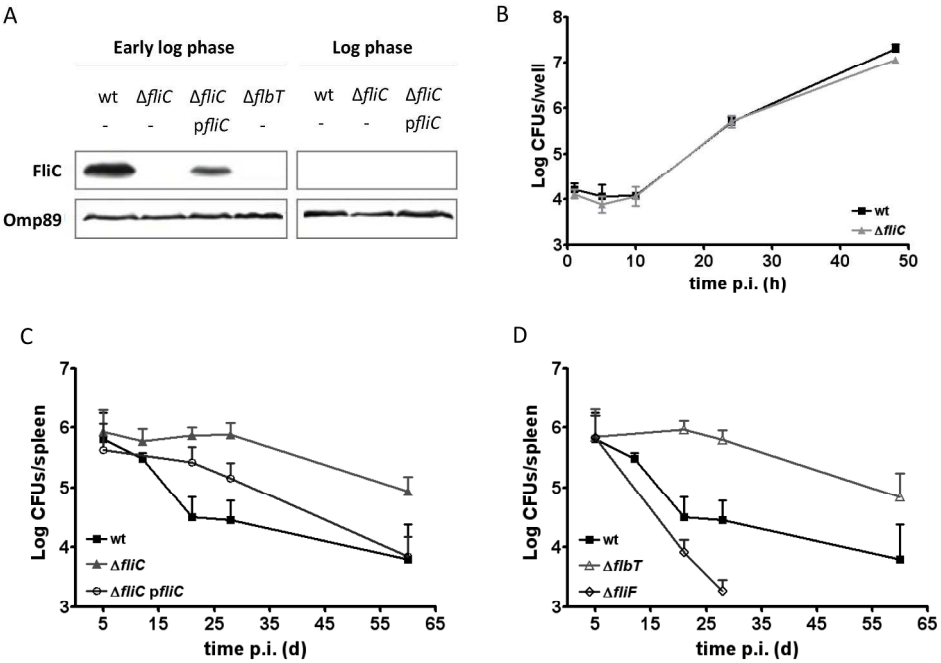

Fig. 1. Flagellin-deficient B. melitensis mutants infect macrophages in vitro with the same kinetics as wt bacteria but show enhanced persistence in mice.
(A) Western blot analysis of the production of flagellin (FliC, upper panel) by B. melitensis strains harvested at the early log phase and the log 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. ΔfliC pfliC is the complemented strain. (B) Intracellular replication of B. melitensis 16M wt and ΔfliC strains in RAW264.7 murine macrophages. Error bars represent the standard deviation of triplicates in one representative experiment out of three. (C) Infection kinetics in the spleens of wt BALB/c mice (n=5) inoculated intraperitoneally (i.p.) with 4 x 104 CFUs of B. melitensis 16M wt, ΔfliC, complemented ΔfliC pfliC (D) Infection kinetics in the spleens of wt BALB/c mice (n=5) inoculated intraperitoneally (i.p.) with 4 x 104 CFUs of B. melitensis 16M wt, ΔflbT, or ΔfliF strains. Data represent the mean CFUs per organ and error bars represent standard deviation. Results have been analyzed 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.

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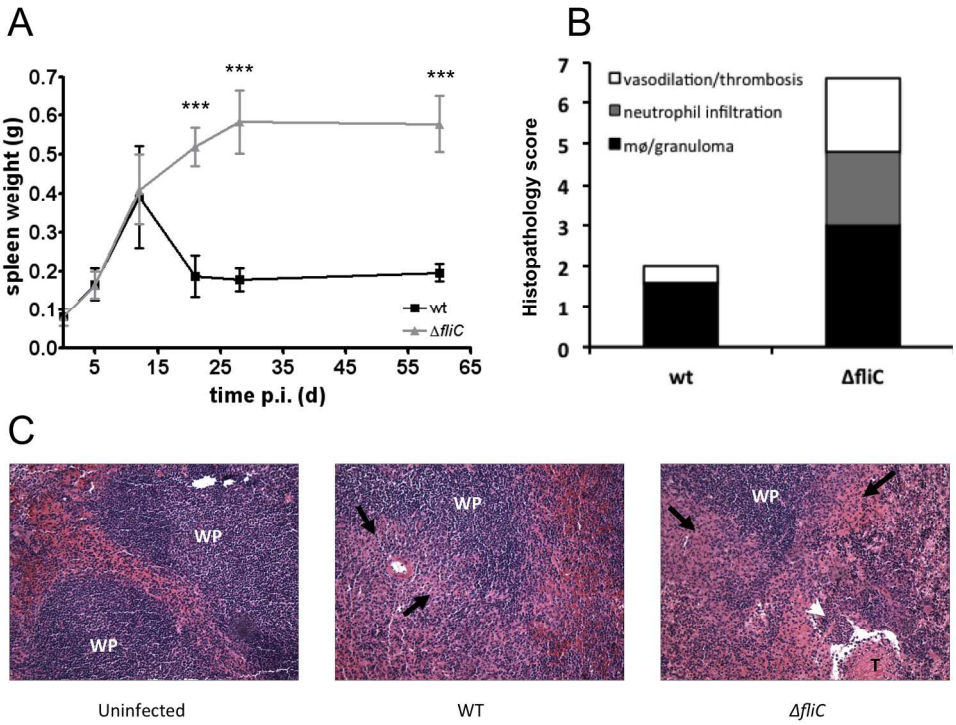

Fig. 2. Enhanced persistence of B. melitensis ΔfliC in mice is associated with increased pathology. (A) Kinetics of splenomegaly in wt female BALB/c mice (n=5) injected i.p. with 4 x 104 CFUs of wt or ΔfliC strains of B. melitensis 16M. Data represent the mean spleen weight and error bars represent standard deviation. Results have been analyzed 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 the Material and methods. Data were analysed using a Mann Whitney test, and the mean histopathology scores were significantly different (P=0.009) (C) Representative photomicrographs (x10) of histopathology of spleens from BALB/c mice uninfected or infected for 28 days with B. melitensis wt or ΔfliC strain. WP, white pulp; T, thrombosis; black arrows, granuloma; white arrowhead, neutrophil infiltration. These results are representative of at least two independent experiments.

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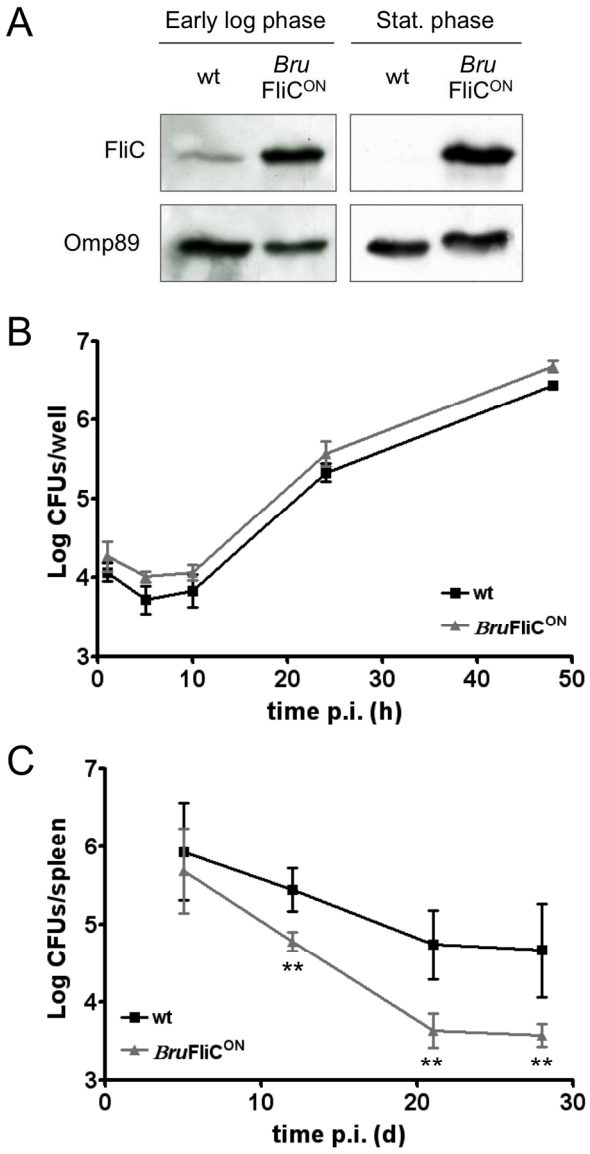

Fig. 3. Constitutive production of flagellin does not impair replication of B. melitensis 16M in macrophages in vitro, but attenuates its virulence in vivo.
(A) Western blot analysis of flagellin (FliC, upper panel) production in wt and BruFliCON strains during early exponential and stationary phases of growth in 2YT rich medium. Detection of Omp89 was used as a loading control. (B) Intracellular replication of wt and BruFliCON 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 x 104 CFUs of wt or BruFliCON strain. Data represent the mean CFUs per organ and error bars represent standard deviation. Results have been analyzed 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.

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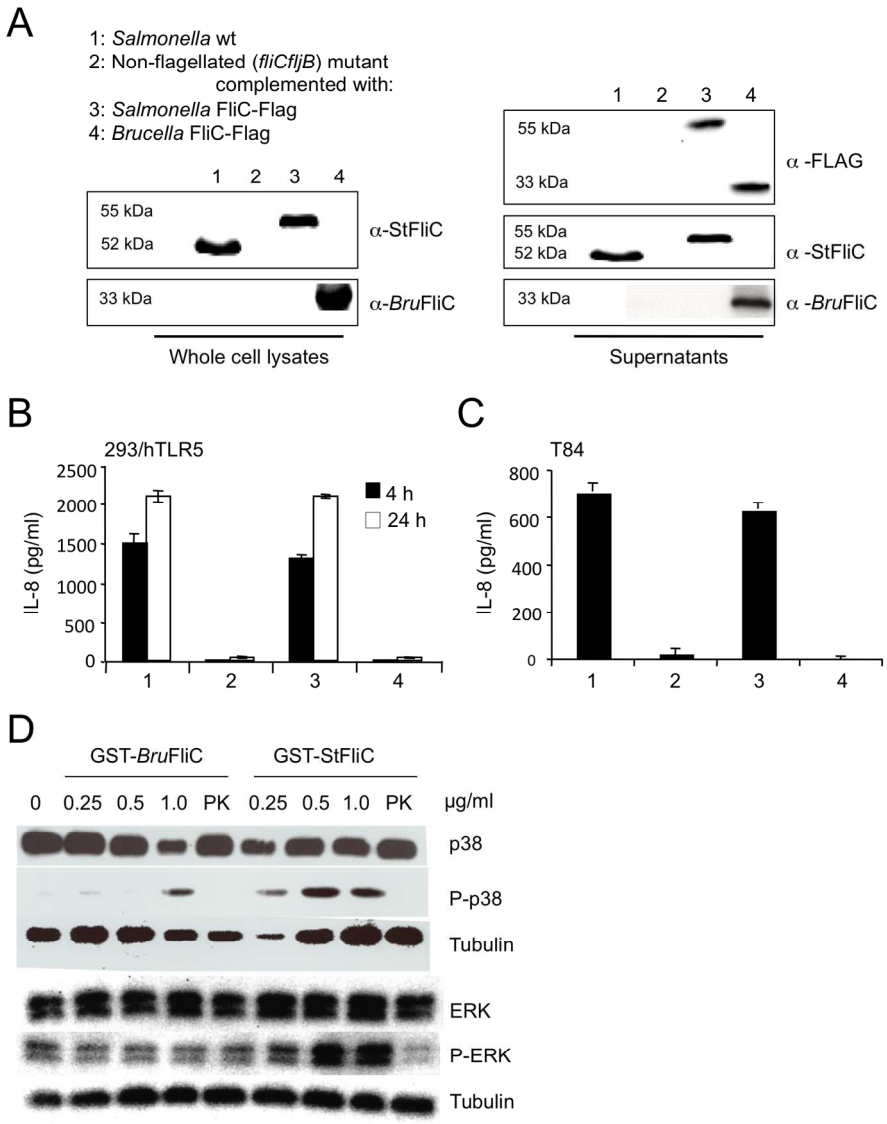

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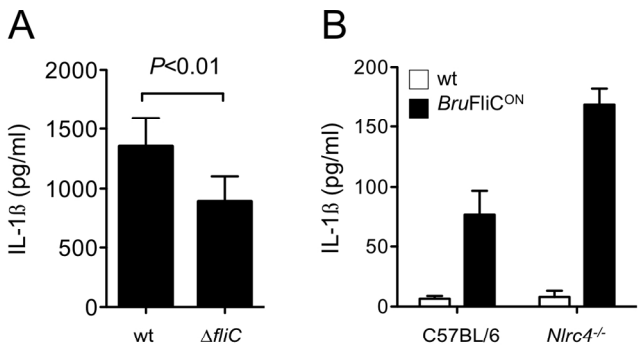

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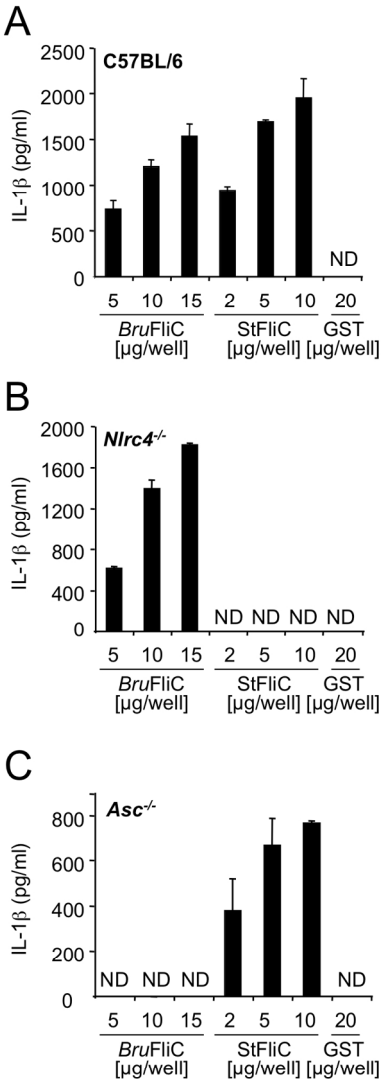

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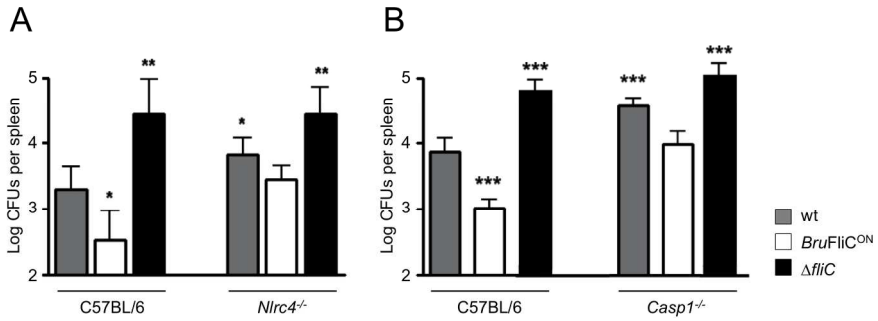

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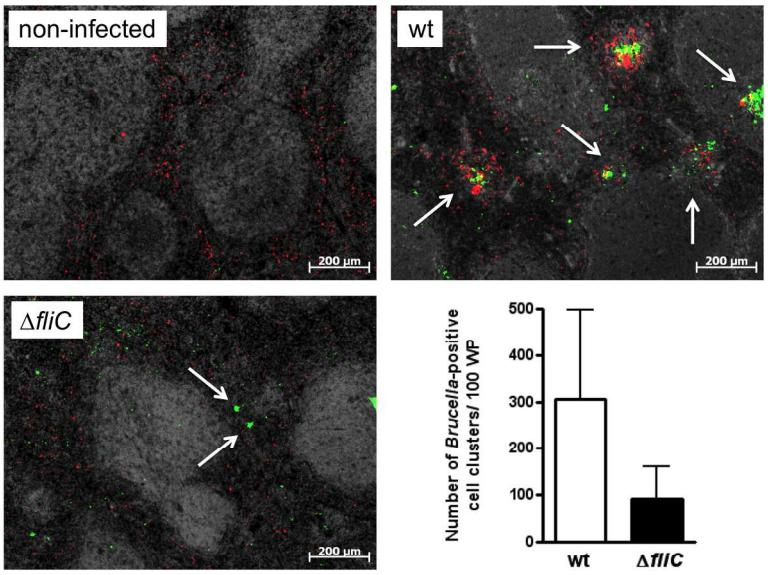

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