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

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Innate immune recognition of flagellin limits systemic persistence of Brucella

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3 **1 Innate immune recognition of flagellin limits systemic persistence of *Brucella***

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48
49 24 **Running title:** *Brucella* flagellin as innate immune signal

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51 25 _____

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2
3 26 **Abstract**
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5 27 *Brucella* are facultative intracellular bacteria that cause chronic infections by limiting innate
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7 28 immune recognition. It is currently unknown whether *Brucella* FliC flagellin, the monomeric
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9 29 subunit of flagellar filament, is sensed by the host during infection. Here, we used two
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11 30 mutants of *Brucella melitensis*, either lacking or overexpressing flagellin to show that FliC
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13 31 hinders bacterial replication *in vivo*. The use of cells and mice genetically deficient for
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15 32 different components of inflammasomes suggested that FliC was a target of the cytosolic
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17 33 innate immune receptor NLRC4, and that the cytosolic adaptor ASC was involved in its
18
19 34 recognition. Accordingly, we showed that FliC was translocated into the cytosol of infected
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21 35 cells. However, our work also suggested that the lack of TLR5 activity of *Brucella* flagellin
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23 36 and the tight regulation of its synthesis and/or delivery into host cells are both part of the
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25 37 stealthy strategy of *Brucella* towards the innate immune system. Nevertheless, since a
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27 38 flagellin-deficient mutant of *B. melitensis* was found to cause histologically demonstrable
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29 39 injuries in the spleen of infected mice, we suggested that recognition of FliC during infection
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31 40 plays a crucial role in the immunologic standoff between *Brucella* and its host, which is
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33 41 characterized by a persistent infection with limited inflammatory pathology.
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43 Introduction

44 The mammalian innate immune system relies on a limited number of pattern recognition
45 receptors (PRRs) to detect microbial-derived molecules during infection and subsequently
46 trigger an appropriate immune response to the invading pathogen. These microbial features
47 are often referred to as PAMPs for pathogen-associated molecular patterns. The PRRs include
48 toll-like receptors (TLRs), which sense PAMPs on the cell surface or in endosomes (Kawai *et*
49 *al.*, 2011), and Nod-like receptors (NLRs), which are cytosolic receptors responding to
50 PAMPs and endogenous danger signals (Lamkanfi *et al.*, 2009, Brodsky *et al.*, 2009a). After
51 stimulus recognition, TLRs initiate multiple signalling pathways involved in the innate
52 inflammatory and antimicrobial responses, as well as in the initiation and control of adaptive
53 immune responses (Kawai *et al.*, 2011). In contrast, upon stimulation, several NLRs,
54 including NLRP1 (also known as NALP1), NLRP3 (NALP3 or cryopyrin), and NLRC4 (Ipaf)
55 assemble inflammasomes, which are multiprotein complexes responsible for activation of the
56 inflammatory cysteine protease caspase-1 (Schroder *et al.*, 2010).

57 Bacterial flagellin, the monomeric subunit of flagellar filament, is a PAMP for both systems.
58 Extracellular flagellin is detected by TLR5 (Hayashi *et al.*, 2001) that activates the MyD88-
59 dependent signalling pathway, leading to the nuclear translocation of NF- κ B, and the
60 activation of mitogen activated protein kinases (MAPK), ultimately inducing the secretion of
61 proinflammatory cytokines and chemokines, such as IL-8 (Gewirtz *et al.*, 2001, Eaves-Pyles
62 *et al.*, 2001, Yu *et al.*, 2003). On the other hand, flagellin injected into the cytoplasm of
63 macrophages through bacterial virulence-associated secretion systems is sensed by NLRC4 in
64 association with NAIP5, another member of the NLR family (Kofoed *et al.*, 2011, Zhao *et al.*,
65 2011). Activation of caspase-1 within the NLRC4 inflammasome leads to the maturation and
66 release of biologically active proinflammatory cytokines IL-1 β and IL-18 (van de Veerdonk *et*
67 *al.*, 2011). Moreover, this inflammasome can trigger a proinflammatory form of cell death

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3 68 known as pyroptosis, (Bergsbaken *et al.*, 2009). Finally, it has been shown that NLRC4 plays
4
5 69 a role in maintaining a normal endosome-lysosome trafficking of phagocytized bacteria
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7 70 within macrophages (Amer *et al.*, 2006, Akhter *et al.*, 2009). There is evidence that both
8
9 71 TLR5 and NLRC4 play a role in controlling *in vivo* infections caused by pathogenic bacteria
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11 72 including *Salmonella enterica* serotype Typhimurium (Feuillet *et al.*, 2006), *Legionella*
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13 73 *pneumophila* (Hawn *et al.*, 2003) and *Pseudomonas aeruginosa* (Feuillet *et al.*, 2006, Franchi
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15 74 *et al.*, 2012). However, bacterial countermeasures to avoid flagellin recognition by the innate
16
17 75 immune system have also been described. *Helicobacter pylori* and *Campylobacter jejuni*
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19 76 escape TLR5 recognition as a result of changes in the amino acid sequence of flagellin
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21 77 (Andersen-Nissen *et al.*, 2005), and it has been suggested that *S. Typhimurium* downregulates
22
23 78 *fliC* expression during macrophage infection to avoid a deleterious strong activation of
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25 79 NLRC4 inflammasome (Cummings *et al.*, 2006, Miao *et al.*, 2010a).

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29 80 *Brucella* spp. are Gram-negative bacteria that cause brucellosis, a zoonosis of worldwide
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31 81 importance. In the natural reservoir hosts, including wild and domestic animals, these
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33 82 intracellular pathogens cause abortion and infertility. Humans are accidental hosts and
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35 83 *Brucella melitensis* and *B. abortus* are the most frequent cause of human infection (Corbel,
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37 84 1997). A key characteristic of *Brucella* infection is its chronic nature. Indeed, animals can
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39 85 remain infected for years, and *Brucella* causes a protracted debilitating disease in untreated
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41 86 humans that can result in serious clinical complications (Young, 1995). As a result,
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43 87 brucellosis has an important economic impact on livestock and remains a major public health
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45 88 concern in endemic countries (Pappas *et al.*, 2006).

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49 89 An important aspect of *Brucella* virulence is its capacity to survive, replicate and persist
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51 90 within infected cells (Atluri *et al.*, 2011). Persistence of *Brucella* within cells relies at least in
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53 91 part on its ability to control the intracellular trafficking of its vacuole in order to avoid
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55 92 lysosomal degradation and to gain access to its replicative niche derived from the
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3 93 endoplasmic reticulum (Anderson *et al.*, 1986). Moreover, the success of *Brucella* lies in its
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5 94 stealthy strategy to cope with the innate immune system. First, the structural features of the
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7 95 *Brucella* envelope allow it to avoid sustained recognition by PRRs and subsequent strong
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9 96 inflammatory responses at the onset of infection (Barquero-Calvo *et al.*, 2007). For example,
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11 97 *Brucella* produces a lipopolysaccharide that signals poorly through TLR4, compared to other
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13 98 bacteria (Lapaque *et al.*, 2006, Barquero-Calvo *et al.*, 2007). In addition, *Brucella* can
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15 99 actively control the inflammatory response by producing a protein that interferes with TLR-
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17 100 dependent signalling pathways (Salcedo *et al.*, 2008, Radhakrishnan *et al.*, 2009, Sengupta *et*
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19 101 *al.*, 2009). Along with the lack of cytotoxicity of *Brucella* for highly parasitized host cells, all
20
21 102 the above-mentioned features could render it less noticeable by the host innate immune
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23 103 system than other pathogens (Gross *et al.*, 2000, Barquero-Calvo *et al.*, 2007, Salcedo *et al.*,
24
25 104 2008). Nonetheless *Brucella* spp. have virulence factors such as a VirB type IV secretion
26
27 105 system (T4SS) (O'Callaghan *et al.*, 1999), cyclic β -1,2-glucan (Briones *et al.*, 2001, Arellano-
28
29 106 Reynoso *et al.*, 2005) and flagellar genes (Fretin *et al.*, 2005) that are required for *Brucella* to
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31 107 persist within its host. Although our previous studies focused on the flagellum and its role in
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33 108 persistent infection, it is unknown whether *Brucella* flagellin, FliC, is sensed by the host
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35 109 during infection. Here, we combined host and pathogen genetic approaches to assess the
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37 110 potential of *Brucella* flagellin to stimulate innate immune responses.
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3 112 **Results**
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5 113 **Mice fail to control infection by flagellin-deficient *B. melitensis* mutants.**
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7 114 In a previous study, insertional inactivation of genes located in the three flagellar loci of *B.*
8
9 115 *melitensis* was reported to result in a marked attenuation of its virulence in mice (Fretin *et al.*,
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11 116 2005). At that time, it was assumed that, as described in enterobacteriaceae, the *fliC* gene was
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13 117 not expressed in mutants of genes encoding basal flagellar structures. However, we recently
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15 118 demonstrated that the flagellar expression hierarchy of *Brucella* is not conventional, since the
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17 119 flagellin subunit is still produced in mutants deficient in the hook or basal body (Ferooz *et al.*,
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19 120 2011). To evaluate the specific impact of the absence of FliC flagellin on the virulence of *B.*
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21 121 *melitensis*, non-polar mutants of *fliC* ($\Delta fliC$) and *flbT* ($\Delta flbT$) (Ferooz *et al.*, 2011) were used
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23 122 to infect murine macrophages and BALB/c mice. The FlbT regulator of *B. melitensis* is
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25 123 specifically required for the production of FliC, most likely by allowing translation of the *fliC*
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27 124 mRNA (Ferooz *et al.*, 2011). Accordingly, flagellin was detected neither in the $\Delta fliC$ nor in
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29 125 the $\Delta flbT$ strain harvested at the early exponential phase of growth, whereas the protein is
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31 126 produced by the isogenic wt strain (Fig. 1A).
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36 127 We first compared the intracellular growth of *B. melitensis* $\Delta fliC$ and $\Delta flbT$ to that of wt
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38 128 bacteria in RAW264.7 murine macrophages. No difference in colony forming units (CFUs)
39
40 129 was detected over a 48-h time course (Fig. 1B). Similar results were obtained in HeLa cells
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42 130 (data not shown). Consistent with a normal multiplication in endoplasmic reticulum-derived
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44 131 vacuoles, both $\Delta fliC$ mutant and its isogenic parental strain were found to replicate within
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46 132 calnexin-positive compartments of HeLa cells at 24h p.i (data not shown).
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49 133 Despite the absence of an obvious role for *Brucella* flagellar genes in cellular models of
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51 134 infection, several reports have shown that they are required for the establishment of a
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53 135 persistent infection *in vivo* (Fretin *et al.*, 2005, Zygmunt *et al.*, 2006). To re-evaluate the role
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55 136 of flagellar proteins *in vivo*, BALB/c mice were infected via the intraperitoneal route with *B.*
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3 137 *melitensis* 16M $\Delta fliC$, $\Delta fliB$ and $\Delta fliF$ non-polar mutants. None of the mutants was
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5 138 significantly attenuated 5 days p.i., as compared with the parental strain (Fig. 1C). Moreover,
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7 139 we could confirm that the basal body protein FliF is required for full virulence. Indeed, the
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9 140 $\Delta fliF$ mutant was attenuated at 3 and 4 weeks p.i. (Fig. 1C). In contrast, the virulence of the
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11 141 $\Delta fliC$ strain was exacerbated when compared to its isogenic parental strain, as $\Delta fliC$ -infected
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13 142 mice presented a higher bacterial load in the spleen from 12 days until 60 days p.i. (Fig. 1C).
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15 143 A higher bacterial count was also observed at the same times in the livers of mice infected
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17 144 with the $\Delta fliC$ mutant (data not shown). Similarly, an enhanced persistence of the $\Delta fliC$ strain
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19 145 in the spleens of the resistant C57BL/6 mice has also been observed (data not shown). The
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21 146 use of a low-copy plasmid carrying *fliC* gene along with its predicted flanking regulatory
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23 147 sequences, which restores regulated production of flagellin in the $\Delta fliC$ strain (Fig. 1A),
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25 148 allowed partial complementation of the phenotype of the newly constructed $\Delta fliC$ mutant at
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27 149 28 days p.i. and full complementation at 60 days p.i. (Fig. 1C). Moreover, we could show that
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29 150 the $\Delta fliB$ mutant had similar infection kinetics than the $\Delta fliC$ strain in the spleen of BALB/c
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31 151 mice (Fig. 1C). This further supports the fact that the apparent inability of the host to control
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33 152 bacterial infection is specifically due to the lack of flagellin production by *Brucella*.
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154 **Mice infected with *B. melitensis* $\Delta fliC$ mutant exhibit severe splenic pathology.**

155 *Brucella* is known to induce splenomegaly in infected hosts. During the course of a *B.*
156 *melitensis* 16M infection in BALB/c mice, the spleen weight increases and peaks around 0.4
157 gr (4-fold the spleen weight of an uninfected mice) at 12 days p.i. Afterwards, the spleen
158 weight decreases but remains twice the normal value until the end of the experiment (Fig.
159 2A). In contrast, we found that the splenomegaly of mice infected with flagellin-deficient
160 mutants, while displaying kinetics similar to those of the wt infection during the first 12 days,
161 continued to increase until 28 days p.i. and reached a plateau of almost 5 or 6 times the

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3 162 normal spleen weight by the end of the experiment (Fig. 2A for $\Delta fliC$, data not shown for
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5 163 $\Delta fliB$). A similar exacerbation of splenomegaly was also observed in C57BL/6 mice at 21
6
7 164 days p.i with the $\Delta fliC$ mutant (data not shown). This was in accordance with the enhanced
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9 165 persistence of the flagellin-deficient mutants in mice (Fig. 1C).

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11 166 We further examined the splenic histopathology of BALB/c mice infected for 28 days with wt
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13 167 or $\Delta fliC$ *B. melitensis* strain. At this time, mice infected with the $\Delta fliC$ strain showed a
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15 168 markedly exacerbated splenic inflammation characterized by increased vasodilation,
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17 169 thrombosis, neutrophil infiltration and granuloma formation (Fig 2B and 2C). In contrast,
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19 170 mice infected for 28 days with wt *B. melitensis* had nearly normal splenic morphology, as
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21 171 compared with non-infected mice.
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27 173 **Ectopic production of flagellin attenuates the virulence of *B. melitensis* in vivo.**

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29 174 Mice apparently fail to control infection caused by *B. melitensis* 16M $\Delta fliC$ or $\Delta fliB$ at late
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31 175 time points. This observation suggests that production of flagellin by *Brucella* somehow
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33 176 influences the course of infection. To further test this hypothesis, we engineered a *B.*
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35 177 *melitensis* 16M strain, designated *BruFliC^{ON}*, that constitutively expresses a plasmid-encoded
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37 178 copy of *fliC* from *Escherichia coli* *Plac*. Western blot analysis confirmed that, while
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39 179 production of flagellin by wt bacteria is only detectable at the early exponential phase of
40
41 180 growth, *BruFliC^{ON}* produced higher levels of flagellin throughout *in vitro* growth (Fig. 3A).

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43 181 Ectopic production of flagellin did not impair the invasion and replication abilities of *Brucella*
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45 182 in macrophages *in vitro* (Fig. 3B). However, we found that the *BruFliC^{ON}* strain was
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47 183 attenuated *in vivo* compared with wt *B. melitensis* 16M. While no difference in splenic
48
49 184 bacterial load was observed between the two strains at 5 days post infection of BALB/c mice,
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51 185 0.5 to 1 log fewer CFU of *BruFliC^{ON}* bacteria were recovered at 12, 21 and 28 days p.i. (Fig.
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3 186 3C). Reduced colonization of *BruFliC*^{ON} was also observed in the liver of infected BALB/c,
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5 187 and similar results were also obtained with C57BL/6 mice (data not shown).
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9 189 ***Brucella* flagellin lacks TLR5 agonist activity**

10 190 The altered virulence of the Δ *fliC* and *BruFliC*^{ON} mutants led us to hypothesize that *Brucella*
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12 191 flagellin is detected by the host in order to mount a protective immune response. To ascertain
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14 192 whether innate immune sensing of flagellin contributes to enhanced control of systemic
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16 193 *Brucella* infection, we first determined whether *Brucella* flagellin possesses agonist activity
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18 194 for TLR5. To this end, epitope-tagged FliC flagellins from *Brucella* (*BruFliC*-FLAG) or *S.*
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20 195 *enterica* serotype Typhimurium (*S.* Typhimurium; *StFliC*-FLAG) were expressed in an *S.*
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22 196 Typhimurium *fliCfljB* mutant (EHW26) lacking endogenous flagellin expression.
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24 197 Immunoblotting with the anti-FLAG antibody demonstrated that both *BruFliC*-FLAG and
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26 198 *StFliC*-FLAG were secreted to the supernatant in similar amounts (Fig. 4A). Addition of the
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28 199 C-terminal FLAG tag to *StFliC* prevents its assembly into flagellar filaments, thereby
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30 200 allowing for a direct comparison of effects of flagellin monomers in the absence of a
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32 201 confounding effect on motility, since strains expressing either *StFliC*-FLAG or *BruFliC*-
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34 202 FLAG were aflagellate and non-motile (data not shown).
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40 203 Culture supernatants of *S.* Typhimurium *fliCfljB* expressing recombinant flagellins were used
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42 204 to treat two TLR5-expressing cell lines: the colonic epithelial cell line T84 and
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44 205 HEK293/hTLR5 (Fig. 4B and 4C). Both cell lines secreted interleukin 8 (IL-8) on infection
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46 206 with strains expressing native or FLAG-tagged *StFliC*, demonstrating that addition of the
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48 207 epitope tag to the C terminus of flagellin did not affect its TLR5 agonist activity. Stimulation
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50 208 of IL-8 secretion was dependent on flagellin in both cell lines, since culture supernatants from
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52 209 the *fliCfljB* mutant elicited little (Fig. 4C) or no (Fig. 4B) IL-8. In contrast to *StFliC*-FLAG,
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54 210 expression of *BruFliC*-FLAG did not elicit IL-8 secretion above the level of the *fliCfljB*
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3 211 mutant. Similar results were obtained when T84 or HEK-293/hTLR5 cells were infected with
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5 212 *S. Typhimurium* strains expressing recombinant flagellins (data not shown). The response to
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7 213 *BruFliC* did not appear to be delayed, since extending the time of the assay to 24h did not
8
9 214 allow detection of a response comparable to that elicited by StFliC-FLAG. As a second
10
11 215 readout for TLR5 signaling, we assayed activation of mitogen-activated protein kinases
12
13 216 (MAPK) p38 and ERK by treatment with purified, GST-tagged flagellins. Phosphorylation of
14
15 217 both p38 and ERK was induced to a greater extent by GST-StFliC than by GST-*BruFliC*, and
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17 218 notably no increase in phosphorylation of ERK could be detected after treatment with GST-
18
19 219 *BruFliC* (Fig. 4D). Taken together, these results demonstrate that compared to *S.*
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21 220 *Typhimurium* flagellin, the ability of *Brucella* flagellin to stimulate TLR5 signaling is greatly
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23 221 reduced.
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223 **Cytosolic sensing pathways detect *Brucella* flagellin during infection of macrophages**

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30
31 224 In addition to TLR5, flagellin that enters the cytosol of host macrophages can be sensed by
32
33 225 the NLRC4/NAIP5 pathway (Kofoed *et al.*, 2011, Zhao *et al.*, 2011). To determine whether
34
35 226 cytosolic pathways could detect flagellin during *Brucella* infection, we first used the TEM-1
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37 227 β -lactamase assay to detect translocation of flagellin into the cytosol of *B. abortus*-infected
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39 228 J774 macrophage-like cells. For these experiments, J774 cells were infected with *B. abortus*
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41 229 2308 expressing either a C-terminally tagged copy of *Brucella* flagellin or an irrelevant
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43 230 protein (GST), from a multi-copy plasmid (pFLAG-TEM1; Sun *et al.*, 2007). While cells
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45 231 infected with *B. abortus* expressing GST::Flag-TEM-1 showed no cytosolic β -lactamase
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47 232 activity (no β -lactamase-positive cells in 4 experiments), 0.94% (range: 0.3-2.1%) of cells
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49 233 infected with *B. abortus* expressing the flagellin fusion protein were β -lactamase positive,
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51 234 suggesting potential access of low amounts of flagellin to the cytosol of *Brucella*-infected
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53 235 cells. Next, we determined whether, in primary macrophages, cytosolic flagellin could
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3 236 stimulate innate immune responses. To this end, we compared the ability of *B. melitensis* and
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5 237 its isogenic $\Delta fliC$ mutant to elicit IL-1 β secretion from primary bone marrow-derived
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7 238 macrophages (BMDM). Compared to *B. melitensis* wt, the $\Delta fliC$ mutant elicited significantly
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9 239 reduced IL-1 β secretion (Fig. 5A). This reduction was not the result of differing numbers of
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11 240 intracellular bacteria of the $\Delta fliC$ mutant, since both the $\Delta fliC$ mutant and wt *B. melitensis*
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13 241 were present in the same numbers (data not shown). This partial reduction in IL-1 β secretion
14
15 242 suggests that recognition of flagellin contributes to activation of the caspase-1 inflammasome.
16
17 243 The mechanism of cytosolic flagellin sensing in the context of intracellular infection was
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19 244 further investigated using the *B. melitensis* FliC^{ON} strain, which expresses flagellin
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21 245 constitutively. This strain, as well as a control carrying the empty plasmid pBBR1MCS, was
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23 246 used to infect immortalized BMDM from mice deficient in NLRC4 (Fig. 5B). Constitutive
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25 247 expression of FliC did not affect the ability of *B. melitensis* to survive intracellularly (Fig. 3B
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27 248 and data not shown). The *Bru*FliC^{ON} strain elicited significantly more IL-1 β secretion than the
28
29 249 control strain, confirming data shown in Fig 5A. While these results suggested that under
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31 250 conditions of flagellin expression, flagellin can be sensed by cytosolic PRRs that lead to
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33 251 activation of caspase-1 and secretion of IL-1 β , NLRC4 was not required for flagellin-
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35 252 dependent stimulation of IL-1 β secretion by BMDM *in vitro*.
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254 ***Brucella* flagellin elicits IL-1 β secretion by a mechanism that is distinct from the** 255 **NLRC4/NAIP5 pathway**

256 Since *B. melitensis* is known to inhibit innate immune signalling (Salcedo *et al.*, 2008,
257 Radhakrishnan *et al.*, 2009, Sengupta *et al.*, 2009) we determined whether purified flagellin,
258 in the absence of other *Brucella* factors, would signal similarly to flagellin expressed during
259 cellular infection. For this purpose, purified GST-*Bru*FliC and GST-StFliC were introduced
260 into the cytosol of BMDM using the cationic lipid DOTAP (Franchi *et al.*, 2006). Both

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3 261 *BruFliC* and *StFliC* elicited dose-dependent secretion of IL-1 β from BMDM from C57BL/6
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5 262 mice when introduced into the cytosol using DOTAP (Fig. 6A). Neither GST, DOTAP alone,
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7 263 nor recombinant flagellins in the absence of DOTAP elicited any secretion of IL-1 β (Fig. 6A
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9 264 and data not shown). Comparison of IL-1 β secreted in response to equal amounts of *StFliC* or
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11 265 *BruFliC* suggested that the proinflammatory activity of *StFliC* was slightly higher than that of
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13 266 *BruFliC* (Fig. 6A). Secretion of IL-1 β in response to *S. Typhimurium* *FliC* was dependent on
14
15 267 NLRC4 and only partially dependent on the adaptor protein ASC (apoptosis-associated speck-
16
17 268 like protein), as reported previously (Broz *et al.*, 2010). In contrast, *BruFliC* elicited IL-1 β
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19 269 secretion that required ASC, but was independent of NLRC4, at least in cultured BMDM
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21 270 (Fig. 6B and Fig. 6C). These results suggested that in BMDM, *Brucella* flagellin was sensed
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23 271 by a cytosolic mechanism that differs from the NLRC4/NAIP5-dependent response to *S.*
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25 272 *Typhimurium* *FliC* (Kofoed *et al.*, 2011, Zhao *et al.*, 2011).
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32 **The cytosolic flagellin-detection pathway is implicated in the control of *B. melitensis***
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34 **infection *in vivo*.**

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36 276 To evaluate the potential impact of caspase-1 inflammasomes on the control of *Brucella*
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38 277 infection *in vivo*, we infected *Nlrc4*^{-/-} and *Casp1*^{-/-} C57BL/6 mice with *B. melitensis* 16M.
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40 278 Splenic bacterial count was examined 21 days p.i., a time at which wt mice manage to
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42 279 effectively control infection caused by flagellin-producing *Brucella* strains (Fig. 1C and 3C).
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44 280 At this time, we observed that NLRC4 (Fig. 7A) and caspase-1 (Fig. 7B) deficiency
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46 281 moderately but significantly affected the resistance of mice to infection. This suggests that the
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48 282 NLRC4-caspase-1 axis is required for the host to control *B. melitensis* 16M infection,
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50 283 possibly through recognition of cytosolic flagellin. To further test this hypothesis, the
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52 284 *BruFliC*^{ON} strain was used to infect *Nlrc4*^{-/-} and *Casp1*^{-/-} mice. As shown previously, virulence
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54 285 of this strain is attenuated compared to wt *B. melitensis* 16M, as the spleen of *BruFliC*^{ON}-
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3 286 infected C57BL/6 mice contained less CFUs than these infected by the wt strain (Fig. 7).
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5 287 Interestingly, this virulence defect was rescued in mice deficient for the cytosolic flagellin
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7 288 sensor NLRC4 (Fig. 7A) or the downstream caspase-1 (Fig. 7B). These data indicate that, in
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9 289 contrast to what has been observed *in vitro* (Fig. 6), *Brucella* flagellin can activate the
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11 290 NLRC4 inflammasome *in vivo*. Nevertheless, although *Nlrc4*^{-/-} and *Casp1*^{-/-} mice infected
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13 291 with wt *B. melitensis* 16M had significantly higher splenic bacterial counts than those of wt
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15 292 mice, it remained significantly lower than those of mice infected with the Δ *fliC* mutant (Fig.
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17 293 7). This suggests that both inflammasome-dependent and inflammasome-independent control
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19 294 of infection operates downstream detection of *Brucella* flagellin *in vivo*.
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25 296 ***B. melitensis* Δ *fliC* mutant fails to elicit early granuloma formation in the spleen of**
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27 297 **infected mice.**

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29 298 Chronic granulomatous inflammation in the spleen of natural hosts, humans and mice is the
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31 299 hallmark of *Brucella* infection (Spink *et al.*, 1949, Enright *et al.*, 1990). Recently, we revealed
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33 300 the pivotal role of early splenic granuloma formation in the ability of mice to control bacterial
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35 301 dissemination (Copin *et al.*, 2012). Here, we used a rabbit polyclonal serum raised against *B.*
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37 302 *melitensis* (anti-*Bru*) with the aim to compare the distribution of putative infected cells in the
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39 303 spleen of BALB/c mice inoculated with *B. melitensis* 16M wt or Δ *fliC* strain. 5 days after
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41 304 infection with *B. melitensis* 16M wt, clusters of cells stained with anti-*Bru* (*Bru*-positive cells)
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43 305 were found equally in white pulp and red pulp area of the spleen (Fig. 8). These clusters
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45 306 consisted primarily of CD11b⁺ cells, suggesting that they corresponded to the granuloma
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47 307 previously described (Copin *et al.*, 2012). Strikingly, at the same time, the number of *Bru*-
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49 308 positive cells clusters counted in splenic sections of Δ *fliC*-infected mice was reduced (Fig. 8).
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51 309 This apparent defect in early splenic granuloma formation suggests the importance of flagellin
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53 310 sensing by the host for the orchestration of this typical tissue response to *Brucella* infection.
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5 312 **Discussion**

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7 313 Intracellular survival and immune evasion both contribute to persistence of *Brucella* in the
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9 314 host (Atluri *et al.*, 2011). Recent studies have shown that *Brucella* uses passive as well as
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11 315 active mechanisms to evade detection by TLRs of the innate immune system (Lapaque *et al.*,
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13 316 2006, Barquero-Calvo *et al.*, 2007, Salcedo *et al.*, 2008, Radhakrishnan *et al.*, 2009, Sengupta
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15 317 *et al.*, 2009). Accordingly, the inflammatory response induced at the onset of *Brucella*
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17 318 infection is lower than observed with pyogenic infections such as salmonellosis (Barquero-
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19 319 Calvo *et al.*, 2007). Actually, brucellae are not entirely invisible to the immune system, which
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21 320 can still detect them and shape a Th1 response to control infection (Murphy *et al.*, 2001,
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23 321 Copin *et al.*, 2007). However, the host immune response is not sufficient to eliminate bacteria,
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25 322 resulting in a chronic state of infection characterized by a balance between pathogen virulence
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27 323 and host resistance. The impact of *Brucella* flagellin on infection had not been reported yet.
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29 324 The data presented here suggest that flagellin plays a crucial role in the interplay between
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31 325 *Brucella* and its host, as its detection by the innate immune system is required for the control
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33 326 of infection *in vivo*, although some characteristics of *Brucella* flagellin would contribute to
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35 327 the stealthy strategy of this pathogen.

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43 329 The use of two mutants of *B. melitensis* 16M that either overproduce or lack the FliC flagellin
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45 330 has shown that this protein hinders bacterial replication *in vivo*. Indeed, a strain engineered to
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47 331 ectopically produce flagellin (*BruFliC^{ON}*) was attenuated in mice, whereas deletion of *fliC*
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49 332 (Δ *fliC*) enhanced persistence of *B. melitensis* 16M in these conditions. Our *in vivo* data are
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51 333 consistent with studies reporting exacerbated infections caused by a flagellin deficient mutant
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53 334 of *Salmonella enterica* serovar Typhimurium (Vijay-Kumar *et al.*, 2006), *Legionella*
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55 335 *pneumophila* (Molofsky *et al.*, 2006) or *Pseudomonas syringae* pv. *Tabaci* (Li *et al.*, 2005),
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3 336 as well as virulence attenuation due to flagellin overproduction by *S. Typhimurium* (Salazar-
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5 337 Gonzalez *et al.*, 2007, Miao *et al.*, 2010a) and *Listeria monocytogenes* (Grundling *et al.*,
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7 338 2004). These findings also suggest that *Brucella* flagellin is an important immune target
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9 339 during infection, and our work provides first insights into the mechanisms involved.

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14 341 TLR5 and the NLRC4/NAIP5 complex are the only proteins currently known as innate
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16 342 immune sensors of extracellular and cytoplasmic bacterial flagellin, respectively (Miao *et al.*,
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18 343 2007).

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21 344 In agreement with a recent paper quoting that purified *Brucella* flagellin does not induce
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23 345 expression of interferon-inducible resistance proteins (IRGs) in murine macrophages
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25 346 (Lapaque *et al.*, 2009), the data reported in this paper allow us to conclude that *Brucella*
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27 347 flagellin is not a TLR5 agonist. This is consistent with its atypical sequence as it lacks the
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29 348 amino acid residues required to stimulate this PRR (Andersen-Nissen *et al.*, 2005). Thus, we
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31 349 propose that *Brucella* evades TLR5-mediated detection, and that it could be part of its stealthy
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33 350 strategy to avoid activation of the innate immune system during the onset of infection.

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36 351 Cytosolic flagellin activates a complex comprising the NLR family proteins NLRC4 and
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38 352 NAIP5 (Franchi *et al.*, 2006, Miao *et al.*, 2006, Kofoed *et al.*, 2011, Zhao *et al.*, 2011). This
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40 353 complex senses a highly conserved region of the C terminal part of the flagellin critical for
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42 354 flagellum filament assembly (Yonekura *et al.*, 2003), but that is required neither for flagellin
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44 355 translocation into the host cell cytosol nor for TLR5 activation (Lightfield *et al.*, 2008). The
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46 356 C-terminal 35 amino acid residues are conserved in *Brucella* FliC flagellin, as they share
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48 357 respectively 46% and 40% identity with *L. pneumophila* FlaA and *S. Typhimurium* FliC, both
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50 358 known to activate NLRC4 (Franchi *et al.*, 2006, Zamboni *et al.*, 2006) and sharing themselves
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52 359 60% identity. Recently, it has been proposed that the minimal motif of flagellin sensed by
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54 360 NLRC4 comprises the highly conserved last C-terminal residues VLSLL found in *L.*

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3 361 *pneumophila* FlaA and *S. Typhimurium* FliC (Lightfield *et al.*, 2008, Miao *et al.*, 2010b).
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5 362 This motif is semi-conserved in *Brucella* flagellin that bears an ILSFR motif.
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7 363 Our results suggest that, similar to what is seen with *L. pneumophila* infection (Amer *et al.*,
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9 364 2006, Case *et al.*, 2009) the NLRC4-caspase-1 axis is involved in the control of *B. melitensis*
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11 365 16M *in vivo* (Fig. 7). However, the absence of NLRC4 or caspase-1 stimulation in mice
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13 366 infected with the flagellin-deficient *B. melitensis* 16M Δ *fliC* or Δ *flbT* mutants cannot by itself
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15 367 account for the inability of the host to control infection. Indeed, the relative difference of
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17 368 virulence between *B. melitensis* 16M wt and Δ *fliC* strains were only partially reduced in
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19 369 *Nlrc4*^{-/-} and *Casp1*^{-/-} mice (Fig. 7), indicating involvement of both NLRC4/caspase-1-
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21 370 dependent and independent mechanisms in the control of *Brucella* downstream flagellin
22
23 371 recognition. This contrasts with what is observed after intratracheal infection of mice with *L.*
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25 372 *pneumophila*. Indeed, in this case, the number of *flaA* mutants and wt bacteria in the lungs of
26
27 373 *Nlrc4*^{-/-} and *Casp1*^{-/-} is similar (Amer *et al.*, 2006, Case *et al.*, 2009). Therefore, it suggests
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29 374 that *Brucella* flagellin is an immune target not only for the cytosolic sensor NLRC4 *in vivo*.
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31 375 Actually, the observation that the *BruFliC*^{ON} strain is still attenuated (a reproducible 0.5 log
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33 376 decreased CFUs in the spleen) compared to *B. melitensis* 16M wt in *Nlrc4*^{-/-} and *Casp1*^{-/-} mice
34
35 377 21 days p.i. is consistent with the hypothesis that *Brucella* flagellin stimulates another
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37 378 immune pathway in addition to the NLRC4/caspase-1 axis. The ASC-dependent signalling
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39 379 suggested by our *ex-vivo* data (Fig. 6) could be this additional pathway. This would be similar
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41 380 to what has been described for *L. pneumophila* that triggers an ASC-dependent activation of
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43 381 caspase-1 in macrophages, in addition to the NLRC4-dependent activation triggered by
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45 382 cytosolic flagellin (Case *et al.*, 2009). Activation of these innate immune pathways by
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47 383 flagellin would play a role in limiting replication of *Brucella in vivo*. However, the immune
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49 384 effector mechanisms involved remain to be uncovered. Processing of the proinflammatory
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51 385 cytokines pro-IL-1 β and pro-IL-18 (Raupach *et al.*, 2006, Dinarello, 2009), pyroptosis
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3 386 (Bergsbaken *et al.*, 2009, Miao *et al.*, 2010a) and control of phagosome maturation (Amer *et*
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5 387 *al.*, 2006, Akhter *et al.*, 2009) that can all result from caspase-1 activation are important
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7 388 processes for innate immunity against bacterial pathogens (Brodsky *et al.*, 2009b).

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9 389 Besides its impact on the innate immune system, it is known that bacterial flagellin is also a
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11 390 target of the adaptive immune response (Salazar-Gonzalez *et al.*, 2005). However, whether
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13 391 the adaptive immune system responds to MHC class II-presented flagellin peptides during
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15 392 infection by *Brucella* is currently not known.
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20 394 While searching for immune effector mechanisms triggered by flagellin detection and
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22 395 involved in the control of *Brucella* replication in mice, we found that the $\Delta fliC$ mutant fails to
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24 396 elicit early granulomatous response in the spleen of mice infected for 5 days, a time at which
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26 397 the mutant is found at a similar level as the wt strain (Fig. 8). Thus, we suggest that detection
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28 398 of flagellin by the host would play a role in early granuloma development during brucellosis.
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31 399 Although the granulomatous response was stronger at 28 days p.i. (Fig. 2B), when the $\Delta fliC$
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33 400 strain colonized spleens at higher extent than wt, an early alteration in this response could
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35 401 contribute to the apparent failure of mice to control infections caused by the flagellin-
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37 402 deficient mutants of *B. melitensis* 16M. Indeed, granulomatous inflammation is the typical
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39 403 tissue response to *Brucella* infection in both mice and humans (Spink *et al.*, 1949, Hunt *et al.*,
40
41 404 1967, Enright *et al.*, 1990), and a recent study has demonstrated the crucial role of early
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43 405 formation of splenic granuloma in the control of *B. melitensis* 16M (Copin *et al.*, 2012).
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45 406 Whether granuloma formation during infection by *Brucella* depends on ASC, NLRC4 and/or
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47 407 caspase-1 is currently unknown. Up to now, a role for the NLRC4 inflammasome in such a
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49 408 response has never been reported. However, it was recently shown that granuloma formation
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51 409 in chronic *M. tuberculosis* infection is dependent on ASC, whereas it does not require
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53 410 caspase-1 (McElvania Tekippe *et al.*, 2010).
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5 412 *S. Typhimurium* translocates flagellin from its containing-vacuole into the cytosol of infected
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7 413 cells by a SPI1-T3SS-dependent but flagellar secretory apparatus-independent process (Sun *et*
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9 414 *al.*, 2007). Similarly, a Dot/Icm T4SS-mediated flagellin translocation has been suggested in
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11 415 the case of *L. pneumophila* (Ren *et al.*, 2006, Molofsky *et al.*, 2006). Here, we show that
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13 416 *Brucella* flagellin is also translocated into the host cell cytosol. Interestingly, flagellin
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15 417 translocation was not seen when a *virB2* mutant was used to infect macrophages (data not
16
17 418 shown), suggesting that VirB T4SS may play a role in flagellin translocation. Interestingly, a
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19 419 requirement for the T4SS to elicit splenic microgranuloma formation has been proposed
20
21 420 (Rolan *et al.*, 2009). According to our results, it could be envisioned that the VirB T4SS of
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23 421 *Brucella* elicits a granulomatous response by translocating flagellin. However, since the T4SS
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25 422 is also essential for *Brucella* to reach its replicative niche (Celli *et al.*, 2003), additional
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27 423 studies would be necessary to determine whether the role of the T4SS in release of flagellin to
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29 424 the host cytosol is direct or indirect. The TEM1 β -lactamase reporter assay has been
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31 425 previously used to demonstrate translocation of *S. Typhimurium* flagellin into the cytosol of
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33 426 infected macrophages (Sun *et al.*, 2007). We observed that the amount of flagellin
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35 427 translocated into cells by *Brucella* is far less than by *Salmonella*. While flagellin could be
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37 428 detected in the cytosol of 77.5% of macrophages infected for 4h with *S. Typhimurium* (Sun *et*
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39 429 *al.*, 2007), less than 1% of cells were positive 16h after infection with *B. abortus*. Therefore,
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41 430 although the intrinsic ability of *Brucella* and *Salmonella* flagellin to induce IL-1 β secretion
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43 431 from BMDM appeared to be similar (Fig. 6), *Brucella* might evade activation of a robust
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45 432 innate immune response from cytosolic PRRs by controlling the production and/or delivery of
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47 433 flagellin into the host cell. Accordingly, we could show that the attenuation of the *BruFliC*^{ON}
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49 434 strain that ectopically produces flagellin is due to a strong NLRC4 inflammasome activation
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51 435 *in vivo* (Fig. 7). Thus, we propose that the tight regulation of flagellin synthesis and/or
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3 436 delivery during infection is part of its stealthy strategy. This has also been suggested for *S.*
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5 437 Typhimurium, which downregulates the expression of *fliC* during macrophage infection
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7 438 (Cummings *et al.*, 2006).
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11 440 In conclusion, we propose that flagellin is an important molecular actor of the interplay
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13 441 between *Brucella* and its host. Although flagellin escapes detection by TLR5 and *Brucella*
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15 442 controls its production and/or delivery to the infected host cell cytosol, its detection by
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17 443 cytosolic PRRs initiates a response that results in an immunologic standoff between *Brucella*
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19 444 and its host, leading to a persistent infection with limited inflammatory pathology. The
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21 445 increased bacterial tissue loads and destructive pathology, seen with the flagellin-deficient
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23 446 mutant demonstrates that innate and possibly also adaptive, recognition of flagellin is a
24
25 447 process that is important to the chronic and stealthy nature of *Brucella* infection. As such,
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27 448 flagellin could be considered as a “host protective factor” (Shames *et al.*, 2010) in the context
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29 449 of brucellosis.
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35 452 **Experimental procedures**

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37 454 **Bacteria and growth conditions**

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39 455 Bacterial strains and plasmids are listed in [Table 1](#). Cultures of *Brucella* strains were freshly
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41 456 inoculated from frozen stock onto 2YT medium (10% yeast extract, 10 g liter⁻¹ tryptone, 5 g
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43 457 liter⁻¹ NaCl) plates before subculturing aerobically at 37°C in 2YT broth supplemented with
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45 458 appropriate antibiotics. LB broth was used for *Escherichia coli* and *Salmonella enterica*
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47 459 serotype Typhimurium (*S.* Typhimurium) cultures. Antibiotics were used at the following
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3 460 concentrations: carbenicillin, 100 mg/liter; chloramphenicol, 30 mg/liter; kanamycin, 60
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5 461 mg/liter; or nalidixic acid, 50 mg/liter.
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10 463 **Molecular techniques**

11 464 DNA manipulations were performed according to standard techniques (Ausubel *et al.*, 1991).

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13 465 Primers used are listed in [Table 2](#).

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16 466 Generation of the complementation vector pRH001-*fliC*: *fliC* coding sequence (cds) and its
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18 467 predicted upstream and downstream regulatory sequences were amplified by PCR using the
19
20 468 *PfliC* and *tfliC* primers pair. The PCR product (*PfliC-fliC-tfliC*) was then cloned into the
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22 469 *EcorRV* site of pGEM. In a second step, this fragment was excised using *BamHI* and *XbaI*,
23
24 470 and inserted into the corresponding sites of pMR10*cat* (R. Roberts, unpublished) in the
25
26 471 opposite orientation to the *Plac*.
27

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29 472 Generation of the *B. melitensis* 16M FliC^{ON} strain: The *fliC* overexpression vector pBBR1-
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31 473 *fliC* was obtained as follows: first, the constitutive promoter of the lac operon *Plac* was
32
33 474 amplified by PCR using the *Plac* and *fliC-Plac* primers pair. In the resulting PCR product,
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35 475 *Plac* is flanked by translation stop codons in all three reading frame in 5' and by the 21st *fliC*
36
37 476 coding sequence (cds) base pairs in 3'. *fliC* cds was amplified by PCR using the *BmfliC-F* and
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39 477 *BmfliC-R* primers. A third PCR using the *Plac* and *BmfliC-R* primers was used to ligate the
40
41 478 two PCR products by cohesive ends. Stop codons and close fusion of *fliC* cds to *Plac* without
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43 479 any linker ensure the production of FliC flagellin that does not bear additional N-terminal
44
45 480 amino acid residues. The PCR product (*XbaI-Plac-fliC-BamHI*) was then cloned into the
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47 481 *EcorRV* site of pGEM. In a last step, this fragment was excised using *XbaI* and *BamHI*, and
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49 482 inserted into the corresponding sites of pBBR1 MCS-I (Kovach *et al.*, 1994) in the opposite
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51 483 orientation to the endogenous *Plac*. This gave rise to pBBR1-*Plac-fliC*. This final
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3 484 construction was transformed into *E. coli* strain S17-1 (Simon *et al.*, 1983), and introduced
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5 485 into *B. melitensis* 16M by conjugation.
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7 486 Generation of C-terminally FLAG-tagged flagellins: A derivative of the broad host range
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9 487 plasmid pBBR1MCS (pBBR1-FLAG) was first generated by ligating a fragment containing
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11 488 “*SphI*-promoter-*NdeI*-*SalI*-3x-Flag-STOP-*PstI*-*SacI*” into pBBR1MCS4 treated with *SphI* and
12
13 489 *SacI*. The *S. Typhimurium fliC* gene was amplified using primers StFliC-F and StFliC-R, and
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15 490 the resulting amplicon was ligated into *NdeI* and *SalI*-digested pBBR1-FLAG to yield
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17 491 plasmid pYHS1116, encoding StFliC-FLAG. The *B. abortus fliC* gene was amplified using
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19 492 primers BaFliC-F and BaFliC-R and cloned in the same way to generate pYHS1073,
20
21 493 encoding BaFliC-FLAG. In both constructs, expression of the recombinant proteins was
22
23 494 controlled by a previously described constitutive *Brucella* promoter, BMEII0193 (Eskra *et*
24
25 495 *al.*, 2001). The constructs were confirmed by DNA sequencing across the junction fragments.
26
27 496 Plasmids pYHS1116 (StFliC-FLAG) and pYHS1073 (BaFliC-FLAG) were introduced into a
28
29 497 *Salmonella fliC fljB* mutant (EHW26, (Raffatellu *et al.*, 2005)) by electroporation. The *B.*
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31 498 *abortus* and *B. melitensis* FliC proteins are identical except for a substitution of Ala156 to Thr
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33 499 in *B. abortus*.
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38 500 Generation of fusions to TEM-1 β -lactamase: To express BaFliC fused with TEM1, *B.*
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40 501 *abortus fliC* was amplified by using the primer pair BaFliC-F and BaFliC-R. The amplicon
41
42 502 was cloned into pCR2.1, then subsequently digested with *NdeI* and *PstI*, and ligated
43
44 503 pFlagTEM1 (Raffatellu *et al.*, 2005) digested with the same enzymes to yield pBaFliCTEM1.
45
46 504 The expression of BaFliC::TEM1 in pBaFliCTEM1 is under the control of inducible Trc
47
48 505 promoter. Constructs expressing StFliC::TEM1 were described previously (Sun *et al.*, 2007).
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51 506 Generation of GST-flagellin fusion proteins: For construction of plasmids expressing GST
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53 507 fused at the N-terminus of flagellins, flagellin genes were amplified to delete predicted N-
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55 508 terminal secretion domains. The *fliC* gene from *S. Typhimurium* was amplified without its
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3 509 first 332 nucleotides using primer pair of StFliC-F2 and StFliC-STOP-R. Similarly *B. abortus*
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5 510 *fliC* lacking its first 87 nucleotides was amplified using primer pair of BaFliC-F2 and BaFliC-
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7 511 R2. Both amplicons were cloned in pCR2.1, excised as *Bam*HI/*Sal*I fragments, and ligated to
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9 512 *Bam*HI/*Sal*I –digested pGEX-4T-1. The cloning junctions were confirmed by DNA sequence
10
11 513 analysis, and the resulting constructs, pGEX-StFliC and pGEX-BaFliC, were transformed into
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13 514 *E. coli* BL-21. Expression of GST::StFliC and GST::BaFliC was induced by IPTG, and the
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15 515 recombinant flagellins were purified using Glutathione-Sepharose 4B (GE Healthcare).
16
17 516 Protein concentration was measured with DC protein assay (BioRad).
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19 517 Construction of plasmids expressing native *S. Typhimurium* and *B. abortus* flagellins: The
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21 518 pSC101 *ori*-based low copy number plasmid pWSK29 (Genbank AF016889) was digested
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23 519 with PvuII and BsaAI to remove the *lac* promoter, *lacZ*□ fragment, and most of the *f1 ori*, as
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25 520 represented by a 4.7kb fragment product (Figure 2a). The 4.7kb plasmid fragment was gel
26
27 521 purified then treated with Antarctic phosphatase (New England Biolabs, NEB). Primers 102
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29 522 and 103 were used to PCR amplify the -134 to -6 region of *S. Typhimurium* LT2, with primer
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31 523 103 adding an XbaI site which serves to replace the -5 to -1 region of *fliC* and overlaps the
32
33 524 translation start site. Primer 103 also contains a multiple cloning site (MCS), adding unique
34
35 525 restriction sites (in the context of PvuII/BsaAI-digested pWSK29) downstream of the XbaI
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37 526 site. Both primer 102 and 103 contain PvuII sites at their 5' ends, so the resulting PCR
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39 527 product was cleaved with PvuII, gel purified, and blunt ligated to the pWSK29 PvuII/BsaAI
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41 528 fragment with Quick T4 DNA Ligase (NEB) and heat shocked into *E. coli* DH5 α . Clones
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43 529 were screened for the loss of an EcoRV site (proper PvuII site ligated), gain of a single BstBI
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45 530 site (one promoter region insertion) and the orientation with the *fliC* promoter facing away
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47 531 from the pSC101 *ori* (the same directionality as the *lac* promoter in pWSK29) was screened
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49 532 for by BglII/PstI double digestion. Clones fitting this description were sequenced using
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3 533 primers 108 and 109, which flank the insertion site in pWSK29, by SeqWright (Houston, TX,
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5 534 USA). An accurate clone was designated pSPN30.
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10 536 **Preparation of concentrated *S. Typhimurium* culture supernatant containing**
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12 537 **recombinant flagellins**

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14 538 *S. Typhimurium* strains were grown for 4 to 5 hours at 37°C with vigorous shaking by
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16 539 diluting an overnight culture 1 to 100 in 20 ml LB broth plus 1 mM IPTG. Once the OD₆₀₀
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18 540 reached 0.8 to 1.2 bacteria were removed by centrifugation at 4000 rpm for 15 min and 12 ml
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20 541 of the resulting supernatant was passed through a 0.45 µm filter and subject to concentration
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22 542 by using an Amicon Ultra-15 with cutoff of 5K (Millipore) followed by a wash with 10 ml
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24 543 PBS. Protein concentration was determined by DC protein assay (BioRad) and SDS-PAGE
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26 544 followed by Coomassie blue stain. The final protein concentration was adjusted to 1 □g/ul.
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32 546 **Generation of rabbit anti-BaFliC serum and Western blot**

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34 547 *B. abortus fliC* (BaFliC) was amplified using primers BaFliC-F and BaFliC-R and cloned into
35
36 548 pET103 in frame with a 6xHis tag. The resulting BaFliC::6xHis fusion protein was produced
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38 549 and purified by using Ni-NTA kit (Qiagen). Rabbit serum against BaFliC was generated by
39
40 550 Antagene (Antagene Inc., Calif.). For detection of secreted BaFliC the supernatant from 1 ml
41
42 551 culture was precipitated using trichloroacetic acid (TCA) and separated on a 12% SDS-PAGE
43
44 552 gel. Proteins were electrotransferred to a polyvinylidene difluoride (PVDF) membrane.
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46 553 BaFliC was detected by using rabbit anti-BaFliC as primary antibody and as goat anti-rabbit
47
48 554 IgG conjugated to horseradish peroxidase (HRP) as secondary antibody. *S. Typhimurium*
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50 555 Phase I flagellin (FliC) was detected using *Salmonella* Hi antiserum (Difco). C-terminal
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52 556 FLAG-tagged *S. Typhimurium* and *B. abortus* flagellins were detected using anti-FLAG
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56 557 monoclonal antibody (1:5000, Sigma) and a goat anti-mouse IgG antibody conjugated to
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3 558 HRP. HRP activity was detected with a chemiluminescent substrate (PerkinElmer Life
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5 559 Sciences). Flagellin produced by *B. melitensis* 16M was detected as described previously
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7 560 (Fretin *et al.*, 2005).
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11 562 **Measurement of TLR5 agonist activity of flagellins**

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14 563 The human colonic epithelial cell line T-84 was cultured in were maintained in Dulbecco's
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16 564 modified Eagle medium (DMEM)-F12 medium (Gibco), containing 1.2 g/liter sodium
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18 565 bicarbonate, 2.5 mM L-glutamine, 15 mM HEPES, and 0.5 mM sodium pyruvate (Gibco),
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20 566 supplemented with 10% fetal calf serum (FCS). The day before assay cells from 1/3 of a 80 to
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22 567 90% confluent T75 flask were seeded per each 24-well plate containing DMEM-F12 and 2%
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24 568 FCS. HEK293 cells were cultured as previously described (Kestra *et al.*, 2010).
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26
27 569 T84 cells in 24-well plates were either infected with 10 μ l of bacteria grown as above or
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29 570 treated by adding 30 μ l of concentrated bacterial culture supernatant and incubated for 4
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31 571 hours at 37°C under 5% CO₂. For the HEK293 stably transfected with human TLR5, cells
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33 572 were grown in 48-well tissue culture plates and infected for 4-48 h with 10 μ l of bacteria
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35 573 grown as described above or treated by adding 10 μ l of concentrated bacteria culture
36
37 574 supernatant and incubated for 8 hours at 37°C under 5% CO₂. Supernatants were aspirated
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39 575 and centrifuged for 10 min at 6,000 rpm to remove residual bacteria and cell debris before
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41 576 measurement of IL-8 concentration by ELISA.
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47 578 **Mitogen-activated protein kinase (MAPK) phosphorylation assay**

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50 579 T84 cells were seeded in six well plates at a density of 4×10^8 cells per well and incubated for
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52 580 24h in DMEM/F12 + 10% FBS. The following day, cells were rinsed with PBS and the
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54 581 medium replaced with serum-free medium. For analysis of MAP kinase phosphorylation,
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56 582 cells were treated with concentrations of GST-BaFliC or GST-StFliC ranging from 250ng/ml
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3 583 to 1 µg/ml. As a negative control, cells were treated with the highest concentration of flagellin
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5 584 (1µg/ml) that had previously been treated with proteinase K (20mg/ml proteinase K for 1h at
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7 585 37°C, then for 10 min at 75°C to inactivate the protease). After 30 and 90 min, cells were
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9 586 lysed 0.1 ml in phosphosafe extraction reagent (Novagen) containing 2.5% protease inhibitor
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11 587 (Sigma) according to the instructions of the manufacturer. The protein concentration was
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13 588 determined using the Micro BCA kit (Pierce). Total protein (0.01 mg) was resolved by SDS-
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15 589 PAGE and transferred to a polyvinylidene fluoride membrane. Primary antibodies were
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17 590 purchased from Cell Signalling Technology, including the following phosphorylation-specific
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19 591 antibodies: p-ERK and p-p38 (Thr180/Tyr182). Secondary antibodies (goat anti rabbit
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21 592 conjugated to horseradish peroxidase) were purchased from Jackson Immunoresearch and
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23 593 used according to the recommendations of the manufacturer. Peroxidase activity was
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25 594 visualized using Immobilon Western Chemiluminescent HRP Substrate (Millipore). For each
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27 595 primary antibody, a separate membrane was used.
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33 34 597 **Detection of flagellin in the cytosol of infected macrophages**

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36 598 The β-lactamase translocation assay was performed as previously described (Sun *et al.*, 2007).
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38 599 Briefly J774A.1 mouse macrophages were seeded in 96-well coverglass bottom plates and
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40 600 infected with *B. abortus* 2308 expressing either a BaFliC::Flag-TEM-1 fusion proteins, or an
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42 601 irrelevant control (Glutathione-S-transferase::Flag-TEM-1) at a multiplicity of infection of
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44 602 500. Plates were centrifuged at 250 g for 5 min at room temperature to synchronize infection.
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46 603 After incubation for 1 hour at 37 °C in 5% CO₂, free bacteria were removed from the cells by
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48 604 three washes with PBS. A volume of 0.2 ml of Dulbecco's modified Eagle's medium
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50 605 supplemented with 10% heat-inactivated fetal bovine serum, 1% non-essential amino acids, 1
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52 606 mM glutamine containing 1 mM IPTG and 100 µg/ml gentamicin was added to each well, and
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54 607 plates were incubated at 37 °C in 5% CO₂. After 16 h, cells were washed once with Hank's
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3 608 balanced salt solution (Invitrogen) and loaded with the fluorescent substrate CCF2/AM
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5 609 (1mM, Invitrogen) for 1.5 h at room temperature using the standard loading protocol
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7 610 recommended by the manufacturer. Fluorescence microscopy analysis was performed using
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9 611 an Axiovert M200 (Carl Zeiss), equipped with a CCF2 filter set (Chroma Technology).
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11 612 Fluorescence micrographs were captured using a Zeiss AxioCam MRC5 and Zeiss AxioVision
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13 613 4.5 software. Images were imported into Adobe PhotoShop for color adjustment. The number
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15 614 of blue cells containing cleaved CCF2/AM was counted visually and expressed as the
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17 615 percentage of total cells in the well. The experiment was performed four times and the result
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19 616 expressed as geometric mean and range of the four experiments.
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618 **Bone-marrow derived Macrophages**

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

623 For assaying inflammasome activation, 24-well microtiter plates were seeded with bone
624 marrow-derived macrophages at a concentration of 2×10^5 cells/well in 0.5 ml of RPMIsup
625 and incubated over night at 37°C in 5% CO₂. For priming of macrophages, cells were treated
626 for 4h before infection with LPS (100 ng/ml), as previously described (Franchi *et al.*, 2006).

627 Inocula of *B. melitensis* 16M were prepared by growing with shaking in TSB for 24h.

628 Bacteria were treated with a non-agglutinating (1:4,000) dilution of anti-*Brucella* rabbit serum

629 (Difco) for 1h at 37 °C, as described (Rolan *et al.*, 2007) then diluted in RPMIsup to a

630 concentration of 4×10^7 CFU/ml. Approximately 2×10^7 bacteria in 0.5 ml of RPMIsup,

631 containing *B. melitensis* 16M wt or its isogenic *fliC* mutant, were added to each well of

632 macrophages. Three independent assays were performed with triplicate samples, and each

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3 633 experiment included control (C57BL/6) macrophages together with macrophages from mutant
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5 634 mice. Microtiter plates were centrifuged at 250 x g for 5 min at room temperature in order to
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7 635 synchronize infection. Cells were incubated for 20 min at 37°C in 5% CO₂, and free bacteria
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9 636 were removed by three washes with phosphate-buffered saline (PBS). RPMI_{sup} plus 50mg
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11 637 gentamicin per ml was added to the wells, and the cells were incubated at 37°C in 5% CO₂.
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13 638 After 1 h, the RPMI_{sup} plus 50µg/ml gentamicin was replaced with medium containing
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15 639 25µg/ml gentamicin. Wells were sampled after infection by aspirating the medium, lysing the
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17 640 macrophages with 0.5 ml of 0.5% Tween-20 and rinsing each well with 0.5 ml of PBS. Viable
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19 641 bacteria were quantified by dilution in sterile PBS and plating on TSA containing appropriate
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21 642 antibiotics.
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27 644 **Liposome-mediated delivery of flagellins to the macrophage cytosol**

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29 645 Recombinant flagellin proteins were delivered to the macrophage cytosol using the cationic
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31 646 lipid DOTAP (Roche), as described previously (Franchi *et al.*, 2006). Briefly, 50 ml of
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33 647 DOTAP was incubated for 30 min in serum-free media with 2 mg of recombinant flagellins
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35 648 purified as described above. After incubation, 3.5 ml serum-free media was added and 500 µl
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37 649 was used to stimulate 1 x 10⁶ macrophages seeded in 24-well microtiter plates for 3h.
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43 651 **Measurement of cytokines**

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45 652 Mouse IL-1β was measured in culture supernatants by enzyme-linked immunoabsorbent
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47 653 assay (ELISA) (R&D Systems). Human IL-8 was detected using an ELISA kit from
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49 654 BioLegend.
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53 656 **Mice**

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3 657 Wild type (wt) BALB/c, wt C57BL/6, C57BL/6 *Nlrc4*^{-/-} (obtained from Dr. VM. Dixit and
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5 658 described in (Mariathasan *et al.*, 2004)) and C57BL/6 *Casp1*^{-/-} (obtained from Dr. R. Flavell
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7 659 and described in (Kuida *et al.*, 1995)) mice were used in this study. They were bred in the
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9
10 660 animal facility of the University of Namur (Belgium). The animal handling and procedures of
11
12 661 this study were in accordance with the current European legislation (directive 86/609/EEC)
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14 662 and in agreement with the corresponding Belgian law "*Arrêté royal relatif à la protection des*
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16 663 *animaux d'expérience du 6 avril 2010 publié le 14 mai 2010*". The complete protocol was
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18
19 664 reviewed and approved by the Animal Welfare Committee of the Facultés Universitaires
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21 665 Notre-Dame de la Paix (FUNDP, Belgium)(Permit Number: 05-558).
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26 667 **Infection of mice**

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28 668 Mice were injected intraperitoneally (i.p.) with 4×10^4 CFUs of *B. melitensis* 16M in 500µl of
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30 669 PBS. Control animals were injected with the same volume of PBS. Infectious doses were
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32 670 validated by plating serial dilutions of the inocula. At selected time intervals, mice were
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34 671 sacrificed by cervical dislocation. Immediately after being killed, spleen and liver were
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36 672 collected for bacterial counts and histopathologic analyses. For bacterial counts, spleens and
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38 673 livers were homogenized in PBS/0.1% X-100 triton (Sigma). Serial dilutions were plated on
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40 674 2YT media plates for enumeration of tissue-associated CFU.
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45 676 **Histology**

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47 677 Spleens were fixed for 24h in Bouin's fixative, dehydrated for 24h in methanol, then
48
49 678 incubated in toluol and finally in warm paraffin prior to paraffin embedding. Sections (5µm)
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51 679 were rehydrated and stained with hemalun, erythrosin and safran. Blinded histopathology
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53 680 scoring for splenic granuloma formation was performed by a pathologist (MX), according to
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3 681 the following criteria. 0, <5% of splenic parenchyma containing granulomas; 1, 5-20%; 2, 20-
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5 682 40%; 3, 40-40%; 4, >60%.

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10 684 **Immunofluorescence microscopy**

11 685 Spleens were fixed for 6h at 4°C in 2% paraformaldehyde (pH 7.4), washed in PBS,
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13 686 incubated overnight at 4°C in a 20% PBS-sucrose solution under agitation, and washed again
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15 687 in PBS. Tissues were embedded in the Tissue-Tek OCT compound (Sakura), frozen in liquid
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17 688 nitrogen, and cryostat sections (10µm) were prepared. Tissues sections were rehydrated in
18
19 689 PBS, then incubated successively in a PBS solution containing 1% blocking reagent
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21 690 (Boeringer) (PBS-BR 1%) and in PBS-BR 1% containing any of the following mAbs or
22
23 691 reagents: DAPI nucleic acid stain, Alexa Fluor 350 phalloidin, M1/70 (anti-CD11b, BD
24
25 692 Biosciences), homemade anti-*B. melitensis* 16M serum (Copin *et al.*, 2012). Slides were
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27 693 mounted in Fluoro-Gel medium (Electron Microscopy Sciences, Hatfield, PA). Labelled
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29 694 tissues sections were visualized under a Zeiss fluorescent inverted microscope (Axiovert 200)
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31 695 equipped with high-resolution monochrome camera (AxioCam HR, Zeiss).

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36 697 **Statistical analysis**

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38 698 ANOVA I was used for data analysis after testing the homogeneity of variance (Bartlett test).
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40 699 Average comparisons were performed by pairwise Scheffe's test. A Mann Whitney test was
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42 700 used for analysis of histopathology scoring. Errors bars represent standard deviation.

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47 702 **References**

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

<u>Designation</u>	<u>Genotype and/or Phenotype</u>	<u>Source or Reference</u>
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 <i>Nal</i> ^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> $\Delta(mrr-hsdRMS-mcrBC)$ $\phi 80lacZ\Delta M15$ $\Delta lacX74$ <i>recA1 araD139</i> $\Delta(ara, leu)7697$ <i>galU galK rpsL(Str^R) endA1 nupG</i>	Invitrogen
DH5 \square	F- <i>mcrA</i> $\Delta(mrr-hsdRMS-mcrBC)$ $\phi 80lac\Delta Z\Delta M15$ $\Delta(lacZYA-argF)U169$ <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> \square pir	(Simon <i>et al.</i> , 1983)
Plasmids		
pCR2.1	TOPO cloning vector	Invitrogen
pUC-KIXX	pUC4::Tn5 KanR	(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-tfliC</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 <i>et al.</i> , 1991)

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944 **Table 2: Primers used in this work**

Primer	Sequence	Restriction site	Application
BaFliC-F	ACCATATG GCTAGCATTCTTACAAACTCGTCG	NdeI	FLAG-tagged BaFliC and BaFliC::FT fusion protein
BaFliC-R	ACTGCAG TTAGCCGCGGAACAGCGACAGGATCGAC	Sall	
StFliC-F	ACCATATG GCACAAGTCATTAATACAAACAGC	NdeI	FLAG-tagged StFliC and StFliC::FT fusion protein
StFliC-R	AGTCGAC TTAACGCAGTAAAGAGAGGACGTTT TGC	Sall	
StFliC-F2	GAATTC ATGGCACAAGTCATTAATACAAACAGC	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	CGGGATCC AATGCCCGGGATCATGTTGATGC	BamHI	complementation plasmid
tfliC	GCTCTAGAT GCCAGACAGGATGTCCGGGC	XbaI	
Plac	GCTCtagA tagAtagAGCGCAACGCAATTAATGTGAG	XbaI	fliC overexpression plasmid
fliC-Plac	GTTTGTAAGAATGCTAGCCATAGCTGTTTCCTGTGTGAAATTG		
BmfliC-F	ATGGCTAGCATTCTTACAAACTCGT		
BmfliC-R	CGGGATCC TTAGCCGCGGAACAGCG	BamHI	

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

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

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11 951 **Fig. 1. Flagellin-deficient *B. melitensis* mutants infect macrophages *in vitro* with the**
12 **same kinetics as wt bacteria but show enhanced persistence in mice.**

13 953 (A) Western blot analysis of the production of flagellin (FliC, upper panel) by *B. melitensis*
14 strains harvested at the early log phase and the log phase of growth in 2YT rich medium.

15 955 Anti-Omp89 detection was used as a loading control (lower panel). Data are representative of
16 two independent experiments. $\Delta fliC$ *pfliC* is the complemented strain. (B) Intracellular
17 956 replication of *B. melitensis* 16M wt and $\Delta fliC$ strains in RAW264.7 murine macrophages.

18 958 Error bars represent the standard deviation of triplicates in one representative experiment out
19 of three. (C) Infection kinetics in the spleens of wt BALB/c mice (n=5) inoculated
20 959 intraperitoneally (i.p.) with 4×10^4 CFUs of *B. melitensis* 16M wt, $\Delta fliC$, complemented $\Delta fliC$

21 960 *pfliC* (D) Infection kinetics in the spleens of wt BALB/c mice (n=5) inoculated
22 961 intraperitoneally (i.p.) with 4×10^4 CFUs of *B. melitensis* 16M wt, $\Delta fliB$, or $\Delta fliF$ strains.

23 963 Data represent the mean CFUs per organ and error bars represent standard deviation. Results
24 964 have been analyzed by ANOVA I after testing the homogeneity of variance (Bartlett). ** and
25 965 *** denote highly significant (p<0.01 and p<0.001 respectively) differences in relation to wt
26 966 infection. These results are representative of at least two independent experiments.

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28 968 **Fig. 2. Enhanced persistence of *B. melitensis* $\Delta fliC$ in mice is associated with increased**
29 969 **pathology.**

30 970 (A) Kinetics of splenomegaly in wt female BALB/c mice (n=5) injected i.p. with 4×10^4
31 971 CFUs of wt or $\Delta fliC$ strains of *B. melitensis* 16M. Data represent the mean spleen weight and
32 972 error bars represent standard deviation. Results have been analyzed by ANOVA I after testing
33 973 the homogeneity of variance (Bartlett). *** denotes highly significant (p<0.001) differences

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3 974 in relation to wt infection. **(B)** Splenic pathology caused by a 28 day-infection was
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5 975 determined using the histopathology scoring system as described in the Material and methods.
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7 976 Data were analysed using a Mann Whitney test, and the mean histopathology scores were
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9 977 significantly different (P=0.009) **(C)** Representative photomicrographs (x10) of
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11 978 histopathology of spleens from BALB/c mice uninfected or infected for 28 days with *B.*
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13 979 *melitensis* wt or $\Delta fliC$ strain. WP, white pulp; T, thrombosis; black arrows, granuloma; white
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16 980 arrowhead, neutrophil infiltration. These results are representative of at least two independent
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18 981 experiments.
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23 **Fig. 3. Constitutive production of flagellin does not impair replication of *B. melitensis***
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25 984 ***16M* in macrophages *in vitro*, but attenuates its virulence *in vivo*.**

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27 985 **(A)** Western blot analysis of flagellin (FliC, upper panel) production in wt and *BruFliC*^{ON}
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29 986 strains during early exponential and stationary phases of growth in 2YT rich medium.
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31 987 Detection of Omp89 was used as a loading control. **(B)** Intracellular replication of wt and
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33 988 *BruFliC*^{ON} strains in RAW264.7 murine macrophages. Error bars represent the standard
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35 989 deviation of triplicates in one representative experiment out of two. **(C)** Infection kinetics in
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37 990 the spleens of wt BALB/c mice (n=5) inoculated i.p. with 4×10^4 CFUs of wt or *BruFliC*^{ON}
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39 991 strain. Data represent the mean CFUs per organ and error bars represent standard deviation.
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41 992 Results have been analyzed by ANOVA I after testing the homogeneity of variance (Bartlett).
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43 993 ** and *** denote highly significant (p<0.01 and p<0.001 respectively) differences in relation
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45 994 to wt infection. These results are representative of at least two independent experiments.
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52 **Fig. 4. *Brucella* flagellin lacks TLR5 agonist activity.**

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54 997 **(A-C)** FLAG-tagged flagellins from *S. enterica* serotype Typhimurium (StFliC) or *Brucella*
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56 998 *abortus* (BaFliC) were expressed in an *S. Typhimurium fliCfljB* mutant, and culture
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3 999 supernatants containing recombinant flagellins were used to treat cells. (A) Western blot
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5 1000 showing production of bacterium-associated flagellins from *S. Typhimurium* wt (lane 1), *S.*
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7 1001 *Typhimurium fliCfljB*mutant (lane 2), *fliCfljB* mutant expressing StFliC-FLAG (lane 3) or
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9 1002 *fliCfljB* mutant expressing BaFliC-FLAG (lane 4). Flagellins were detected both in the pellets
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11 1003 (left panel) and in the concentrated supernatants (right panel) of *S. Typhimurium* strains. 30ng
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13 1004 of concentrated supernatant proteins from *S. Typhimurium* strains expressing recombinant
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15 1005 flagellins were used to treat HEK293/hTLR5 cells for 4 or 24h (B) and T84 cells for 8h (C).
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17 1006 IL-8 in cell supernatants was measured by ELISA. (D) Activation of p38 and ERK MAPK in
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19 1007 T-84 cells by purified recombinant flagellins from *Brucella* (GST-BaFliC) and *S.*
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21 1008 *Typhimurium* (GST-StFliC) was measured by Western blot analysis with anti- p38, anti-
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23 1009 phosphorylated (P-)p38, anti- ERK, and anti-P-ERK. Detection of tubulin was used as a
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25 1010 loading control. Purified flagellins treated with proteinase K (PK) were used as a control. All
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27 1011 data shown are from an individual experiment that was repeated at least twice with similar
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29 1012 results.
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36 1014 **Fig. 5. *B. abortus* flagellin can enter the cytosol of infected macrophages and induces IL-**
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38 1015 **1 β in an NLRC4-independent manner. (A)** Bone marrow-derived macrophages from
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40 1016 C57BL/6 mice were inoculated with *B. melitensis* 16M wt or the $\Delta fliC$ mutant and IL-1 β was
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42 1017 measured in the culture supernatants by ELISA at 24h p.i. Results are shown as the mean \pm
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44 1018 standard deviation of data from five independent experiments. (B) Bone marrow-derived
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46 1019 macrophages from C57BL/6 or *Nlrc4*^{-/-} mice were inoculated with *B. melitensis* 16M wt or
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48 1020 the *BruFliC*^{ON} strain. IL-1 β in the supernatant was measured at 6h after inoculation. Data
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50 1021 shown are combined from three independent experiments with triplicate samples, and
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52 1022 represent the mean \pm standard deviation of all data.
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3 1024 **Fig. 6. Introduction of recombinant *Brucella* flagellin into the host cell cytosol results in**
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5 1025 **ASC-dependent, but NLRC4-independent secretion of IL-1 β .**

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7 1026 Graded amounts of GST-BaFliC and GST-StFliC fusion proteins were delivered to the
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9 1027 cytosol of LPS-primed bone marrow-derived macrophages from C57BL/6 (A), *Nlrc4*^{-/-} (B) or
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11 1028 *Asc*^{-/-} (C) mice, using the cationic lipid DOTAP. Treated macrophages were incubated for 3h
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14 1029 before measurement of IL-1 β in the supernatants by ELISA. Results are expressed as the
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16 1030 mean of triplicate samples, with error bars representing the range of the data from one of two
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18 1031 independent experiments with the same outcome.
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23 1033 **Fig. 7. NLRC4 inflammasome is implicated in the control of *B. melitensis* infection in**
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25 1034 ***in vivo*.** Wild type, *Nlrc4*^{-/-} (A) and *Casp1*^{-/-} (B) C57BL/6 mice (n=5) were injected i.p. with 4 x
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27 1035 10⁴ CFUs of *B. melitensis* wt, *BruFliC*^{ON} or Δ *fliC* strain, as indicated in the figure. Mice were
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29 1036 sacrificed 21 days post-infection and CFUs per spleen were determined. These results are
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31 1037 representative of at least two independent experiments. Data have been analysed by ANOVA
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33 1038 I after testing the homogeneity of variance (Bartlett). * and ** denote respectively significant
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35 1039 (p<0.05) and highly significant (p<0.01) differences in relation to C57BL/6 wt infection by wt
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37 1040 bacteria.
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43 1042 **Fig. 8. The distribution of *Bru*-positive cells is different in the spleen of mice infected by**
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45 1043 **the Δ *fliC* mutant, compared to wt infection.**

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47 1044 Localization of *Bru*⁺ cells (green) and CD11b⁺ cells (red) in the spleen of BALB/c mice non-
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49 1045 infected or infected with *B. melitensis* wt or the Δ *fliC* strain. The graph represents the relative
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51 1046 number of clusters of *Bru*⁺ cells. Errors bars are the standard deviation calculated on
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53 1047 countings of four mice from two independent experiments.
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3 1049 **Acknowledgements**
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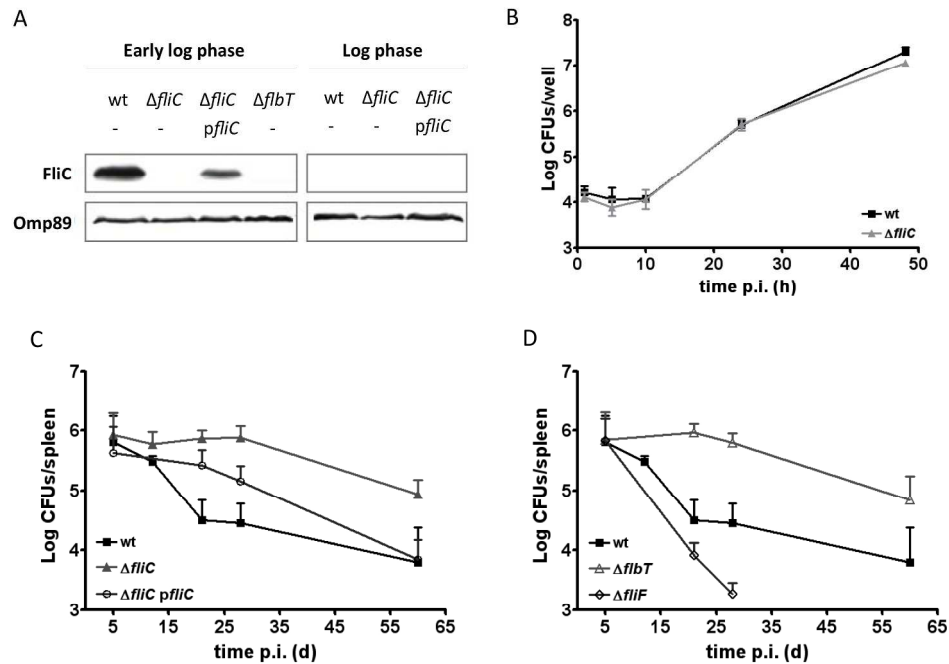


Fig. 1. Flagellin-deficient *B. melitensis* mutants infect macrophages in vitro with the same kinetics as wt bacteria but show enhanced persistence in mice.

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

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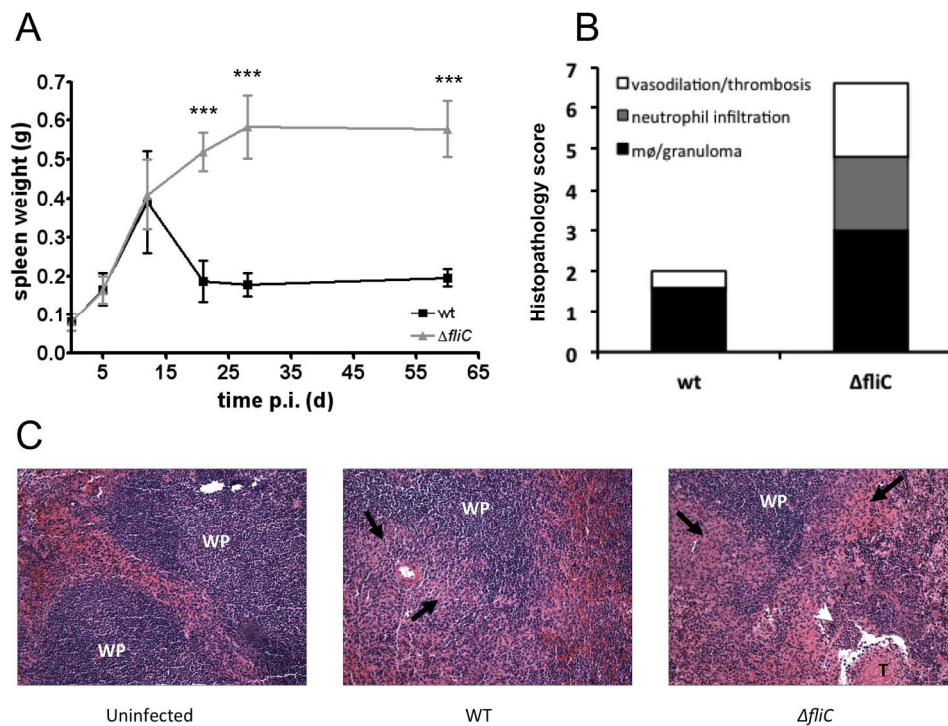


Fig. 2. Enhanced persistence of *B. melitensis* $\Delta fliC$ in mice is associated with increased pathology. (A) Kinetics of splenomegaly in wt female BALB/c mice (n=5) injected i.p. with 4×10^4 CFUs of wt or $\Delta fliC$ strains of *B. melitensis* 16M. Data represent the mean spleen weight and error bars represent standard deviation. Results have been 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 $\Delta fliC$ strain. WP, white pulp; T, thrombosis; black arrows, granuloma; white arrowhead, neutrophil infiltration. These results are representative of at least two independent experiments.

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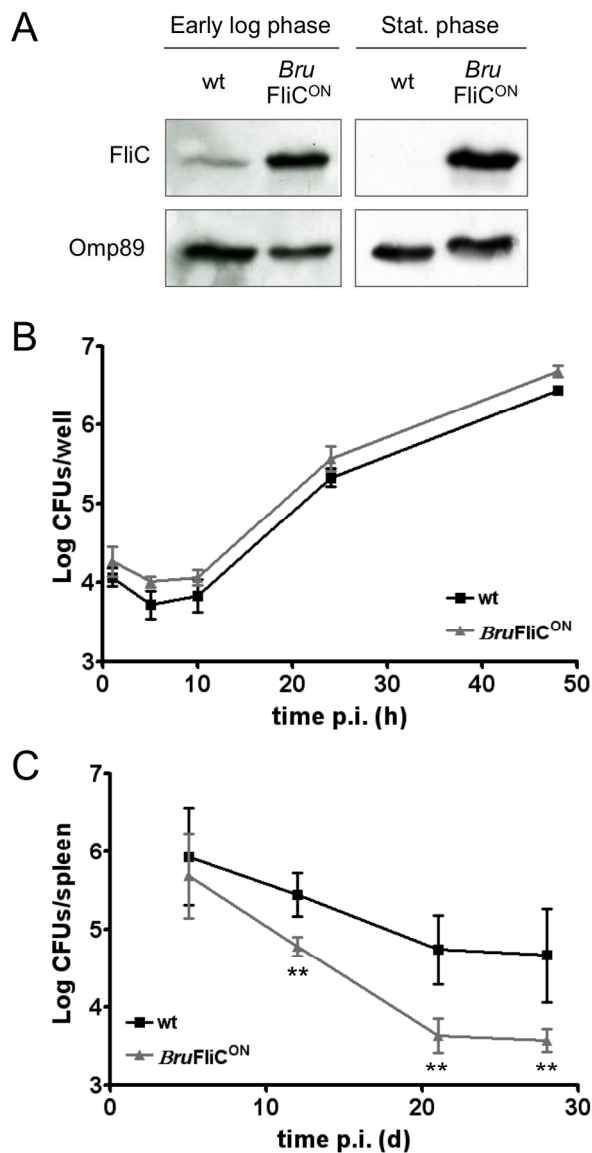


Fig. 3. Constitutive production of flagellin does not impair replication of *B. melitensis* 16M in macrophages in vitro, but attenuates its virulence in vivo.

(A) Western blot analysis of flagellin (FliC, upper panel) production in wt and 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 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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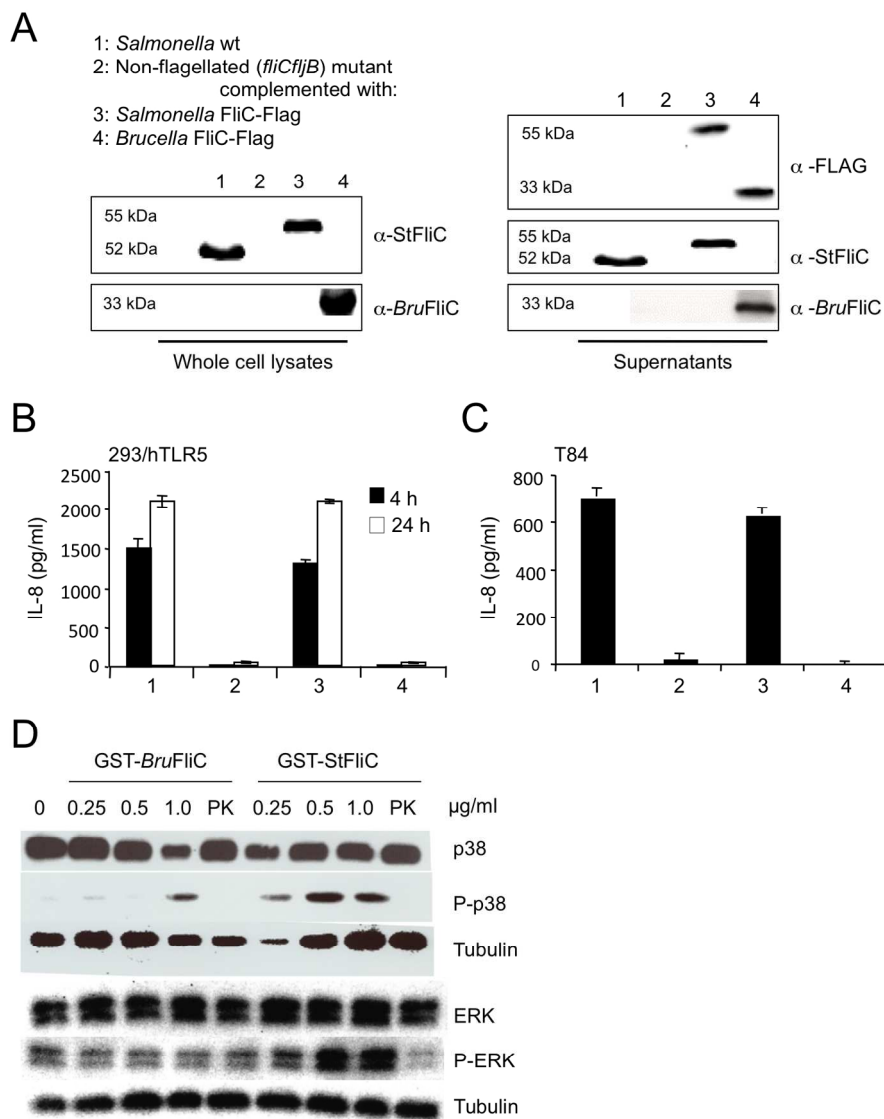


Fig. 4. *Brucella* flagellin lacks TLR5 agonist activity.

(A-C) FLAG-tagged flagellins from *S. enterica* serotype Typhimurium (StFliC) or *Brucella abortus* (BaFliC) 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

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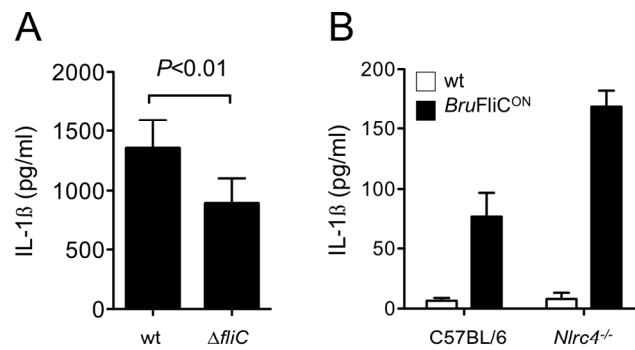


Fig. 5. *B. abortus* flagellin can enter the cytosol of infected macrophages and induces IL-1 β in an NLRC4-independent manner. (A) Bone marrow-derived macrophages from C57BL/6 mice were inoculated with *B. melitensis* 16M wt or the Δ *fliC* mutant and IL-1 β was measured in the culture supernatants by ELISA at 24h p.i. Results are shown as the mean \pm standard deviation of data from five independent experiments. (B) Bone marrow-derived macrophages from C57BL/6 or *Nlr4*^{-/-} mice were inoculated with *B. melitensis* 16M wt or the *BruFliCON* strain. IL-1 β in the supernatant was measured at 6h after inoculation. Data shown are combined from three independent experiments with triplicate samples, and represent the mean \pm standard deviation of all data.

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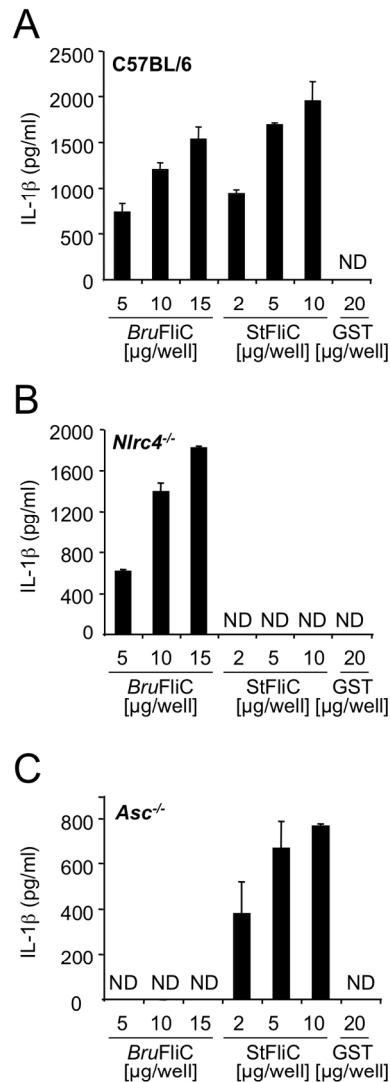


Fig. 6. Introduction of recombinant Brucella flagellin into the host cell cytosol results in ASC-dependent, but NLRC4-independent secretion of IL-1 β .

Graded amounts of GST-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-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.

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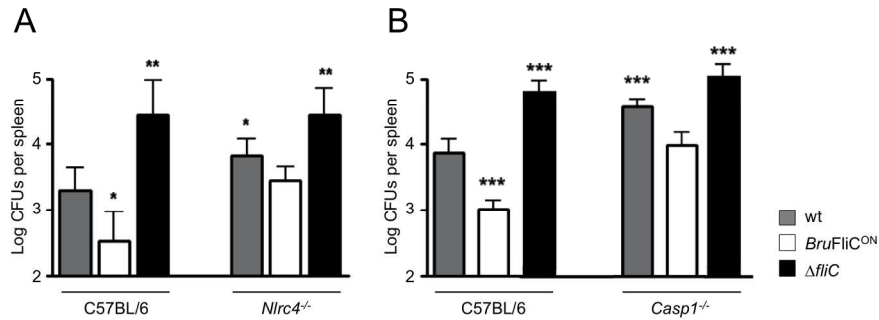


Fig. 7. NLRC4 inflammasome is implicated in the control of *B. melitensis* infection in vivo. Wild type, *Nlrc4*^{-/-} (A) and *Casp1*^{-/-} (B) C57BL/6 mice (n=5) were injected i.p. with 4 x 10⁴ CFUs of *B. melitensis* wt, *BruFliCON* or Δ *fliC* strain, as indicated in the figure. Mice were sacrificed 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.

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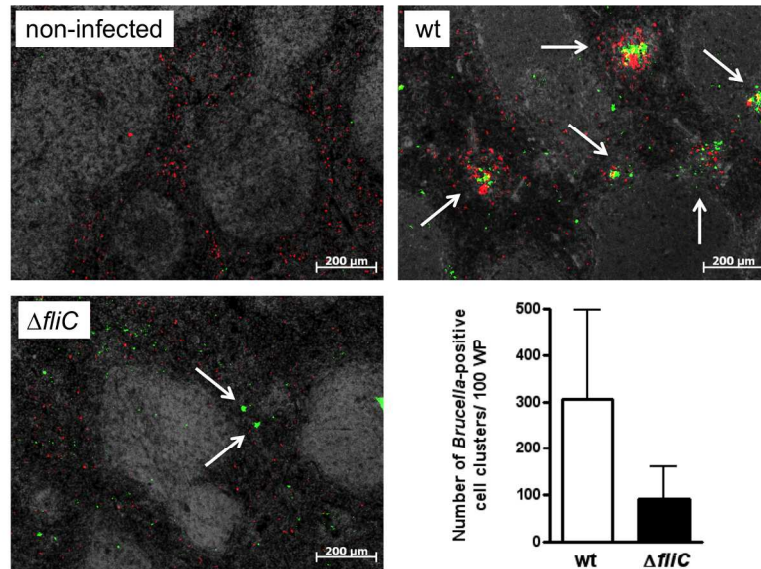


Fig. 8. The distribution of Bru-positive cells is different in the spleen of mice infected by the Δ fliC mutant, compared to wt infection.

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.

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