Brucella abortus induces Irgm3 and Irga6 expression via Type-I IFN by a MyD88-dependent pathway, without the requirement of TLR2, TLR4, TLR5 and TLR9.

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Abstract

Innate immune system senses bacterial pathogens by pattern recognition receptors, such as the well-characterised Toll-like Receptors (TLR). Activation of TLRs signalling cascades depends on different adaptors, which among them MyD88 plays a key role in triggering innate immune responses. Here, we show that in murine macrophages, *Brucella abortus* triggers IRGs (p47 GTPase) expression via type-I IFN secretion at late time points, when *Brucella* has reached its replication niche. This induction requires the adaptor molecule MyD88 but does not involved the TLRs normally implicated in sensing Gram-negative bacteria, namely TLR2, TLR4, TLR5 and TLR9. Interestingly, IRGs upregulation was not detected after stimulation with pure *Brucella* flagellin, ruling out a possible involvement of TLR5. Importantly, *Brucella* mutants lacking the functional VirB type IV secretion system were not capable of inducing Irgm3 and Irga6 expression, suggesting that the type IV secretion system is involved in such process. Our data suggest that *Brucella* is recognized intracellularly by an unknown receptor, different to the conventional ones used for Gram-negative sensing, that signals through MyD88 and that this recognition is mediated by the type IV secretion system.

Introduction

Brucellosis is one of the world's most widespread zoonotic diseases, causing abortion and infertility in mammals resulting in substantial losses in livestock. The causative agent is an intracellular bacteria, *Brucella abortus* transmissible to humans where it can lead to undulant fever, endocarditis, arthritis and osteomyelitis (Franco *et al.*, 2007). Its virulence depends on its ability to infect, survive and replicate in different cell-types (Gorvel and Moreno, 2002). The type-IV secretion system called (virB) has been largely implicated in *Brucella* virulence (Celli *et al.*, 2003; Comerci *et al.*, 2001).

Immune defence against *Brucella* requires $CD4^+$ and $CD8^+$ T cells from the adaptive system (for review see (Golding *et al.*, 2001)). However, the innate immune system has a prominent role as the first line of defence, implicating the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs). Activation of such receptors activates intracellular signalling leading to the secretion of inflammatory cytokines, interferons (IFN), upregulation of antigen presenting and costimulatory molecules. Two well-characterised PRRs classes are the intracellular receptors from the nucleotide-binding oligomerization domain (NOD) family and the transmembrane receptors, Toll-like Receptors (TLRs). Recognition of PAMPs by TLRs stimulates the activation of intracellular signalling pathways via TIR-domain-containing adaptors, including MyD88, TIRAP, TRIF and TRAM (for review see (Kawai and Akira, 2009; Medzhitov, 2007).

In the literature, there are some contradictions about the involvement of TLR2 and TLR4 in the resistance against *Brucella* (Oliveira *et al.*, 2008). However, MyD88 KO mice are susceptible to *Brucella* infection, suggesting a crucial role of this signalling

adaptor in the immune responses against this bacterium (Macedo *et al.*, 2008; Weiss *et al.*, 2005).

The role of type-I IFN has extensively been studied during viral infection and it has been shown that type-I IFN plays a predominant role in defence against viruses. Recently, several studies showed that this cytokine is expressed in responses to bacterial infections (for review, see (Decker *et al.*, 2005)). However, effects of type-I IFN on bacterial load or host resistance against bacteria are controversial. Some studies suggested a detrimental effect of type-I IFN on bacterial infection whereas some other found no effect or even an increased bacterial load (Reutterer *et al.*, 2008; Auerbuch *et al.*, 2004; O'Connell *et al.*, 2004; Freudenberg *et al.*, 2002; Rothfuchs *et al.*, 2001; Cooper *et al.*, 2000). We previously showed that *Brucella* is able to induce type-I IFN expression *in vitro* (Salcedo *et al.*, 2008). The use of IFNα/β knock-out mice seemed to not show any role of this cytokine during *Brucella* infection (Roux *et al.*, 2007). However, type-I IFN is responsible for the induction of crucial proteins of the innate immune system, the members of the IRG (immunity-related GTPases or p47 GTPases) family (for review see (Howard, 2008; Taylor *et al.*, 2007). IRG-deficient mice permit to assess that most of the members of this family are involved in IFN-mediated host response against intracellular pathogens including Gram-negative bacteria such as *Chlamydia* and *Salmonella* (Al-Zeer *et al.*, 2009; Henry *et al.*, 2007).

We previously published that *Brucella* LPS induces IRGs expression via type-I IFN in a TLR4 and MyD88-independent manner (Lapaque *et al.*, 2006). Here, we show an unique pathway of Irgm3 and Irga6 induction via type-I IFN. This pathway includes the adaptor molecules MyD88 but none of TLRs susceptible to recognize Gram-negative

bacteria. More importantly, we show here that the *Brucella* type IV secretion system plays an important role in the activation process and we propose that *Brucella* recognition occurs in the cytoplasm of infected macrophages.

Results

Brucella induces Irgm3 and Irga6 expression, at late time points post-infection, through Type-I interferon

We first investigated kinetics of IRG expression upon *Brucella* infection and we observed that IRG induction of expression was detectable after 24h post-infection (Fig. 1A). Interestingly, Irgm3 expression was detectable not only in the infected cells but also in neighboring cells (Fig. 1B). Supernatants from *Brucella*-infected cell culture were able to inducing Irgm3 expression (Fig. 1C). These results suggested that *Brucella* might be acting through an indirect pathway. As we previously described that IRG induction of expression by LPS, including *Brucella* LPS, depends on the secretion of type-I IFN (Lapaque *et al.*, 2006), we tested whether this would be the case upon *Brucella* infection. To this end, murine macrophages were stimulated with IFN-α or IFN-γ, or infected with *Brucella abortus* for 24h, in the presence or the absence of neutralizing antibodies specific for type-I IFNs and assayed for the induction of Irgm3 expression. Anti-type-I interferon antibodies specifically inhibited Irgm3 expression (Fig. 1D). We concluded that IRG expression is induced indirectly by *B. abortus* through a type-I IFN-dependent pathway.

Brucella does not Induce Irga6 and Irgm3 expression in MyD88-deficient mice

We previously highlighted that *B. abortus* LPS activates a modest IRG expression

through type-I IFN in a MyD88-independent pathway compared to IRG expression level during *Brucella* and *E. coli* infections or upon *E. coli* LPS stimulation (Fig. 2 and (Lapaque *et al.*, 2006)). We then wondered if MyD88 was involved in such a process. We infected macrophages from MyD88 knockout and wild-type mice with *Brucella abortus*. The pattern of strong induction of Irgm3 expression by *E. coli* LPS (5 nM lipid A), IFNs and weak induction by *B. abortus* LPS (at 50 nM lipid A) was maintained in both wild-type and MyD88-deficient mice (Fig. 2). In contrast to wild-type macrophages, MyD88-deficient murine cells failed to respond to *Brucella* infection. In contrast to LPSs and *E. coli* infection, *B. abortus* did not induce IRGs in the absence of MyD88 (Fig. 2 and data not shown). Therefore, Irgm3 and Irga6 induction by *B. abortus* is MyD88-dependent.

Induction of Irga6 and Irgm3 is TLR2-,TLR4-,TLR5- and TLR9-independent

MyD88 is involved in Irgm3 and Irga6 induction by *B. abortus*. This adaptor has a key role in TLR-induced signaling. *Brucella* is a Gram-negative bacteria, which possesses different PAMPs potentially recognized by TLR family members, namely lipoproteins (Omp16 and Omp19; TLR2 ligands), LPS and BLS (TLR4 ligands), flagellin (potentially TLR5 ligand) and *Brucella*'s DNA (TLR9 ligand). To investigate the pathway of *Brucella*-dependent induction of IRG expression, we exploited peritoneal macrophages from TLR2-, TLR4-, TLR5- and TLR9-deficient mice. As controls, we used macrophages from background-matched wild-type mice.

As shown in Fig 3A, the TLR2-ligand PAM_3CSK4 induced Irgm3 expression in a

TLR2-dependent manner whereas Irgm3 *Brucella*-dependent expression was unchanged. Similar results were found using TLR4 and TLR9-deficient mice. Whereas LPSs and CpG ODN failed to induce IRGs expression in TLR4- and TLR9-deficient mice respectively, Irgm3 and Irga6 activation was maintained in response to *Brucella* infection (Fig. 3B and C). To investigate the role of *Brucella* flagellin in IRGs activation, peritoneal macrophages were stimulated with *Salmonella typhimurium, Bacillus subtilis* and *Brucella abortus* purified flagellins (Fig3 D). In contrast to *S. typhimurium* and *B. subtilis* flagellins, *B. abortus* flagellin did not induce IRG expression. Consistent with the above result, both in wild type and TLR5 knockout macrophages, *B. abortus* induced IRGs expression (Fig. 3E). Therefore, Irgm3 and Irga6 induction by *B. abortus* seems to be TLR2-, TLR4-, TLR5- and TLR9-independent.

Induction of Irga6 and Irgm3 is virB-dependent

VirB type-IV secretion system (T4SS) plays a crucial role in *Brucella* intracellular replication. In particular, T4SS is required for the fusion between the endoplasmic reticulum and the *Brucella*-containing vacuole (Celli *et al.*, 2003; Comerci *et al.*, 2001). Since virB T4SS system is able to secrete effector proteins able to modify host cell functions, we investigated if virB was involved in IRG activation. We took advantage of two *virB* mutants, *virB9* and *virB10*. As compared to wild type strain, *virB9* mutant induced low levels of Irgm3 expression in infected murine macrophages (Fig. 4A). This slight induction was presumably in response to *B. abortus* LPS present on bacteria since peritoneal macrophages from TLR4-deficient mice did not express any

Irgm3 upon infection with *virB10* mutants (Fig4. B). These findings suggest that *Brucella* type IV secretion system is involved in the MyD88-dependent induction of IRGs expression.

Experimental procedures

Antibodies and reagents

Rabbit anti-Irga6 (IIGP) antibody n°165 was raised against Irga6/IIGP protein as described (Uthaiah *et al.*, 2003). Irgm3 (IGTP, clone 7) monoclonal antibody was from Transduction laboratories (Lexington, USA). Monoclonal anti-actin (clone AC-40) was from Sigma (St Louis, USA). Secondary antibodies: anti-rabbit and anti-mouse IgG-peroxidase conjugate antibodies were from Sigma (St Louis, USA); mouse Alexa 568 was from Molecular Probes. Neutralizing anti-type-I IFN, recombinant human IFN-α, IFN-β and IFN-γ were from R and D Systems (Minneapolis, USA). *E. coli* and *B. abortus* LPSs were purified as previously described (Lapaque *et al.*, 2006). PAM_3CSK4, CpG ODN, *Salmonella typhimurium, Bacillus subtilis* flagellins were from invivogen. Pure *Brucella* flagellin was a kind gift from JJ. Letesson (Namur, Belgium)

Mice

Seven to eight-week-old female C57Bl/6 mice were purchased from Jackson ImmunoResearch (West Grove, PA). TLR2-, TLR4-, , TLR9- and MyD88-deficient mice were from Dr. Shizuo Akira's laboratory, Osaka, Japan (Hemmi *et al.*, 2000; Hoshino *et al.*, 1999; Kawai *et al.*, 1999; Takeuchi *et al.*, 1999). TLR5-deficinet mice were generated as previously described (Feuillet *et al.*, 2006). Background-matched control wild-type and deficient mice were bred at Centre d'Immunologie de Marseille-Luminy (France).

Macrophage preparation

Four days after intraperitoneal injection of mice with 4% sterile fluid thioglycollate, peritoneal exudate macrophages were extracted by washing the peritoneal cavity with cold Dulbecco's modified Eagle's medium (DMEM) (Gibco). The cells were then washed and resuspended in DMEM supplemented with 10% FCS, 10 mM HEPES, 10 mM sodium pyruvate, 10 mM non-essential amino acids, 2 mM glutamine (Life Technologies), plated and incubated for 2–4 h at 37°C, 5% CO_2. Non-adherent cells were removed from wells or dishes by extensive washes, and the macrophages were then incubated in fresh medium. Macrophages were incubated with LPSs (0.5 or 50 mM), IFN-α (1000 U ml^{-1}), IFN-β (1000 U ml^{-1}), IFN-γ (5–10 U ml^{-1}), flagellins (10, 50 or 100 ng ml^{-1}) or CpG ODN (5μM) or infected with *Brucella abortus* strains during 24 h. Macrophages were lysed with 1% Nonidet 40 in PBS including protease inhibitors, and the protein concentration of each lysate was evaluated with a BCA protein assay kit (Pierce, USA).

Brucella infection

The bacterial strains used in this study were smooth virulent *B. abortus* strain 2308 (Pizarro-Cerda *et al.*, 1998), and the isogenic mutants *virB9*$^{-}$ and *virB10*$^{-}$ infections (Celli *et al.*, 2005; Celli *et al.*, 2003) were performed at a multiplicity of infection of 1:50 for all experiments. Bacteria were centrifuged onto macrophages at 400g for 5 min at 4 °C and incubated 30 min at 37°C with 5% CO_2 atmosphere. After washes, the cells were incubated for 1h in medium supplemented with 100μg ml^{-1}gentamycin. Then, the antibiotic concentration was decreased to 10μg ml^{-1} for the remaining of the experiment.

Western blotting

Equal protein amounts of each sample were run on a 10-12% SDS-PAGE gel and transferred onto Immobilon-P membranes (Millipore, MA). Membranes were blocked in PBS/5% skim milk/0.1% Tween-20 (Sigma) and primary and secondary Abs were successively added in this buffer, for 2 h before developing with the enhanced chemoluminescence system ECL (GE Healthcare).

Confocal microscopy

Immunofluorescence studies were performed as described previously with macrophages grown on coverslips (Lapaque *et al.*, 2006). Briefly, cells were fixed in 3% paraformaldehyde at room temperature for 12 min and then processed for immunofluorescence labelling. Coverslips mounted in Mowiol (Sigma) were viewed under a Zeiss LSM 510 laser scanning confocal microscope. Confocal images were then assembled and analysed using Adobe Photoshop.

Discussion

During infection, the first line of defence is the recognition of PAMPs by PRRs including TLRs and NODs. However, some bacteria evolved with their host in order to circumvent this recognition by displaying unconventional PAMPs. *Brucella* is believed to be amongst them, because it possesses a poor endotoxic lipopolyssacharide (LPS) and a poor activating flagellin ((Lapaque *et al.*, 2005) and fig 3). Therefore, better understanding PRRs and the signal pathways involved in innate immune responses against *Brucella* is of great interest.

In addition to our previous findings, here we demonstrate that *Brucella* is able, via type-I IFN secretion, to induce IRGs expression in macrophages through a MyD88-dependent (via TLR4 recognition of its LPS) and a MyD88–independent pathways ((Lapaque *et al.*, 2006) and the present study). The identity of the receptor(s) recognizing *Brucella* upstream MyD88 remained to be found as we ruled out TLR2, TLR4, TLR5 and TLR9. Such results support previous studies showing that MyD88 was crucial for clearance of *Brucella in vivo*, whereas TLR2/4 were not required (Weiss *et al.*, 2005). However, even if we cannot rule out TLR7 and TLR8, it is unlikely that these receptors are involved is such process as these molecules are known to signal in responses to viral PAMPs (Kawai and Akira, 2007). We also ruled out the MyD88-dependent signalling through IL-1-Receptor, as IL-1 was not able to induce IRGs expression (data not shown). A pathway, involving MyD88 and an unknown PRR recognizing various Gram-positive bacteria have been previously reported including *Streptococcus* and *Bacillus anthracis* (Gratz *et al.*, 2008; Glomski *et al.*, 2007; Henneke *et al.*, 2002). However, as far as we

know, activation of a MyD88-dependent, TLR2/4/5/9-independent signalling pathway is quite unique in Gram-negative bacteria.

The induction of IRGs expression was detectable at late time-point where *Brucella* is already in its replication niche. Our most striking finding is that IRGs expression induced by *Brucella* was dependent of the presence of a functional type-IV secretion system (TT4S) as *virB* mutants were unable of inducing IRG expression. Presumably, this process might implicate the translocation of a bacterial component or effector, which will be recognized by a PRR in the cytoplasm of the infected cells. Roux et al, showed by comparing mice transcriptional profiles after infection with the wild-type or *virB*-deficient strains that TT4S was involved in the induction of type-I and type-II-dependent genes (Roux *et al.*, 2007). Supporting our findings, they found that amongst these genes induced, *in vivo*, by *Brucella* expressing a functional type IV secretion system, members from the IRG family, including Irgm3 (IGTP) and Irga6 (IIGP), were highly represented (Roux *et al.*, 2007). Taking together, these findings suggest that *Brucella* can release into the cytosol via its TT4S a PAMP, which signals through MyD88.

Which PRR and PAMP are involved? Our findings are consistent with previous studies of T4SS-dependent cellular activation. This included the recognition by the NOD family members of the *Legionella pneumophila* flagellin and *Helicobacter pylori* peptidoglycan translocated via T4SS (Molofsky *et al.*, 2006; Viala *et al.*, 2004). The identity of the PAMP and PRR, potent implication of members of the NOD family and, importantly, the link with the adaptor molecule MyD88 remained to be elucidated.

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Figure legends

Fig 1. *Brucella abortus* induce indirectly IRGs expression via type-I IFN at late time point.

A) Irgm3 and Irga6 expression by peritoneal macrophages from wild-type mice stimulated with *E. coli* LPS, IFN-γ (5 Units ml^{-1}), IFN-β (1000 Units ml^{-1}), DMEM medium (control) or after infection with *B. abortus* 2308. Macrophage lysates were collected 12 and 24 h post-infection, and equal protein amounts of each lysate were analyzed for Irgm3 and Irga6 expression by western blotting.

B) Confocal images showing the expression of Irgm3 in infected-macrophages (*B. abortus* in green, Irga6 in red) or in IFN-γ stimulated-macrophages.

C) Peritoneal macrophages from wild type mice were activated with IFN-β (1000 U ml^{-1}), or infected with *B. abortus* (left panel) for 24h. The supernatant of each condition were harvested and add to fresh macrophages for 8h (Left panel). Macrophage lysates were collected at the indicated time, and equal protein amounts of each lysate were analyzed for Irgm3 expression by western blotting.

D) Peritoneal macrophages from wild type mice were activated with IFN-α (1000 U ml^{-1}), IFN-γ (10 U ml^{-1}) or infected with *B. abortus* (Right panel) for 24h. Anti-type I IFN neutralizing antibodies were added where indicated by a (+) sign. Macrophage lysates were collected and analyzed for Irgm3 expression by western blotting.

Fig 2. Induction of IRGs by LPS is dependent on MyD88.

Peritoneal macrophages from wild-type C57/Bl6 (WT) and MyD88-deficient mice were stimulated for 24 h with IFN-γ (5 Units ml^{-1}), IFN-β (1000 Units ml^{-1}), *B. abortus* LPS (50 nM), *E. coli* LPS (0.5 nM) or after infection with *B. abortus* or *E. coli*. DMEM

medium was used as a control (control). Irgm3 expression was assessed by western-blotting.

Figure 3. Induction of IRGs expression by *B. abortus* is independent on TLR2, TLR4, TLR9 and TLR5.

A) Peritoneal macrophages from WT and TLR2 knockout mice were stimulated for 24 h with IFN-γ (5 Units ml^{-1}), *E. coli* LPS (0.5 nM) or TLR2 ligand: Pam_3CSK4 (100 ng $ml^{-1)}$ or infected with *B. abortus*. DMEM medium was used as a control (control). Irgm3 expression was assessed by western-blotting. B) As in A, macrophages from WT and TLR4 knockout mice were stimulated for 24 h with *B. abortus* LPS (50 nM), *E. coli* LPS (0.5 nM), IFN-γ (5 Units ml^{-1}), or infected with *B. abortus*. DMEM medium was used as a control (control). Irgm3 expression was assessed by western-blotting. C) As in A, macrophages from WT and TLR9 knockout mice were stimulated for 24 h with the TLR9 ligand: CpG ODN (5μM), IFN-β (1000 Units/ml), or infected with *B. abortus*. DMEM medium was used as a control (control). Irga6 expression was assessed by western-blotting. D) Peritoneal macrophages were stimulated for 24h with different concentrations (a, 10; b, 50 or c, 100 ng ml^{-1}) of flagellin from *Salmonella typhimurium, Bacillus subtilis,* and *Brucella* or with IFN-β (1000 U ml^{-1}). DMEM medium was used as a control (control). Irga6 expression was assessed by western-blotting. E) As in A, macrophages from WT and TLR5 knockout mice were stimulated for 24 h with IFN-γ (10 Units ml^{-1}), or infected with *B. abortus*. DMEM medium was used as a control (control). Irga6 expression was assessed by western-blotting.

Fig 4. Type-IV secretion system compounds, virB9 and virB10, are required for the *B. abortus*-induced IRGs expression.

A) Irgm3 expression by peritoneal macrophages after infection by *B. abortus* 2308, *VirB9* mutant at different multiplicity of infection (a 25:1; b 50:1; c 100:1) was assessed by western-blot. DMEM medium was used as a control (control).

B) Peritoneal macrophages from TLR4-mutants or wild-type (WT) mice were stimulated for 24h with IFN-γ at 10 Units ml^{-1} or with *E. coli* 0.5 nM lipid A or infected with *B. abortus* or *virB10* mutant. Macrophage lysates were collected and equal protein amounts of each lysate were analyzed for Irgm3 expression by western blotting.

Acknowledgements

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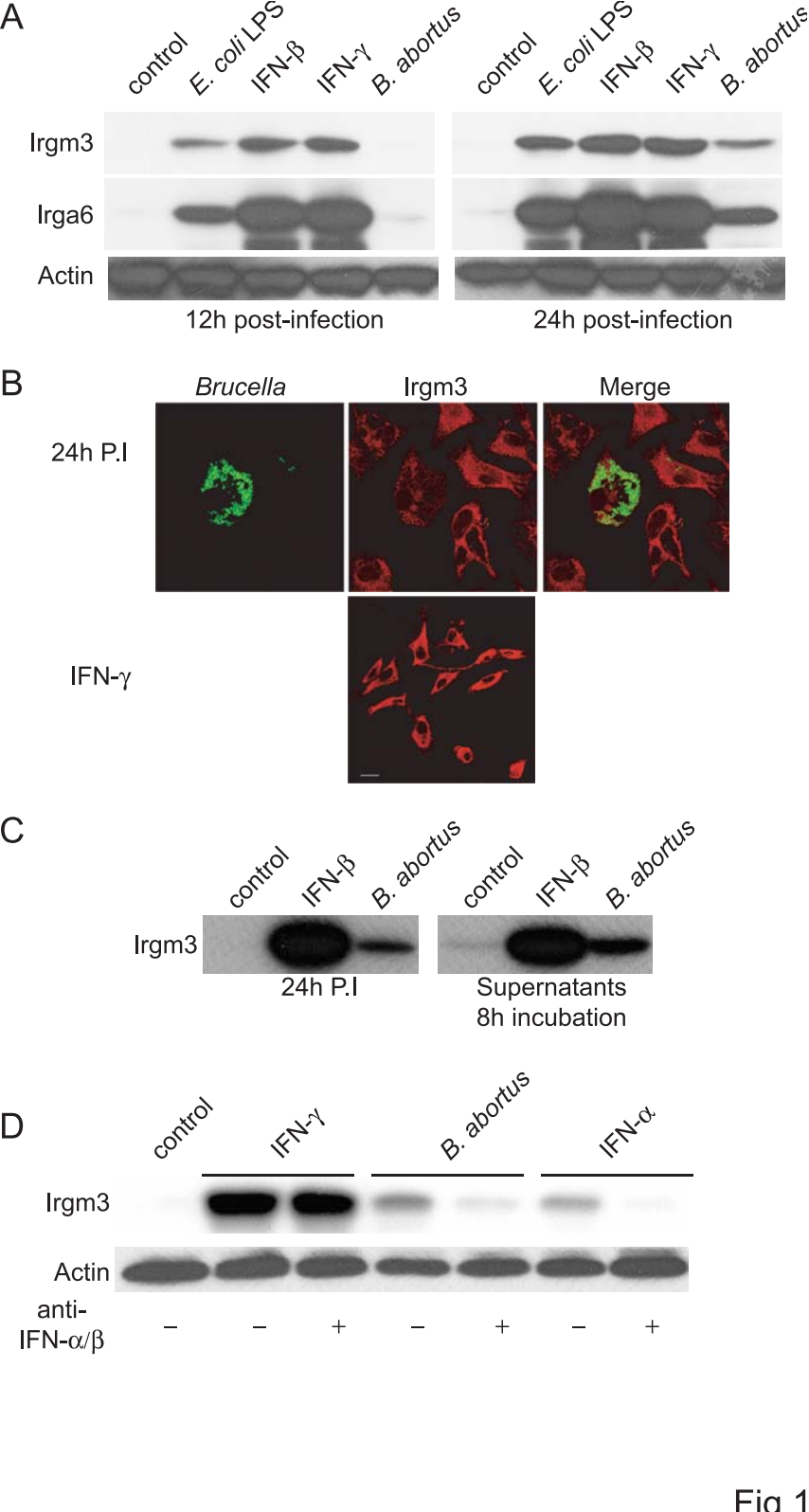

A
control
E. coli LPS
IFN-β
IFN-γ
B. abortus
control
E. coli LPS
IFN-β
IFN-γ
B. abortus
Irgm3
Irga6
Actin
12h post-infection
24h post-infection
B
Brucella
Irgm3
Merge
24h P.I
IFN-γ
C
control
IFN-β
B. abortus
control
IFN-β
B. abortus
Irgm3
24h P.I
Supernatants
8h incubation
D
control
IFN-γ
B. abortus
IFN-α
Irgm3
Actin
anti-
IFN-α/β
– – + – + – +

Fig 1

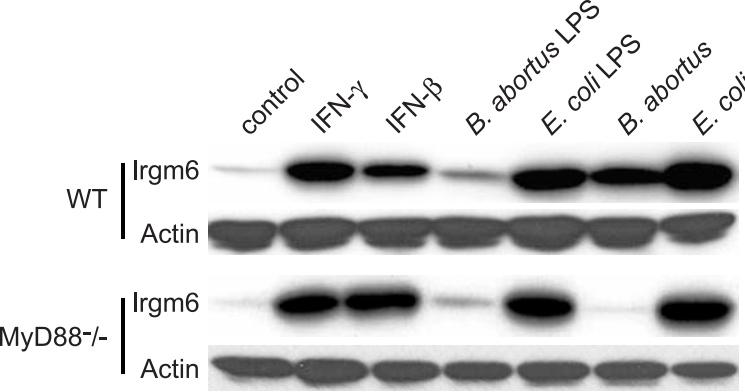

control
IFN-γ
IFN-β
B. abortus LPS
E. coli LPS
B. abortus
E. coli
WT
Irgm6
Actin
MyD88-/-
Irgm6
Actin

Fig 2

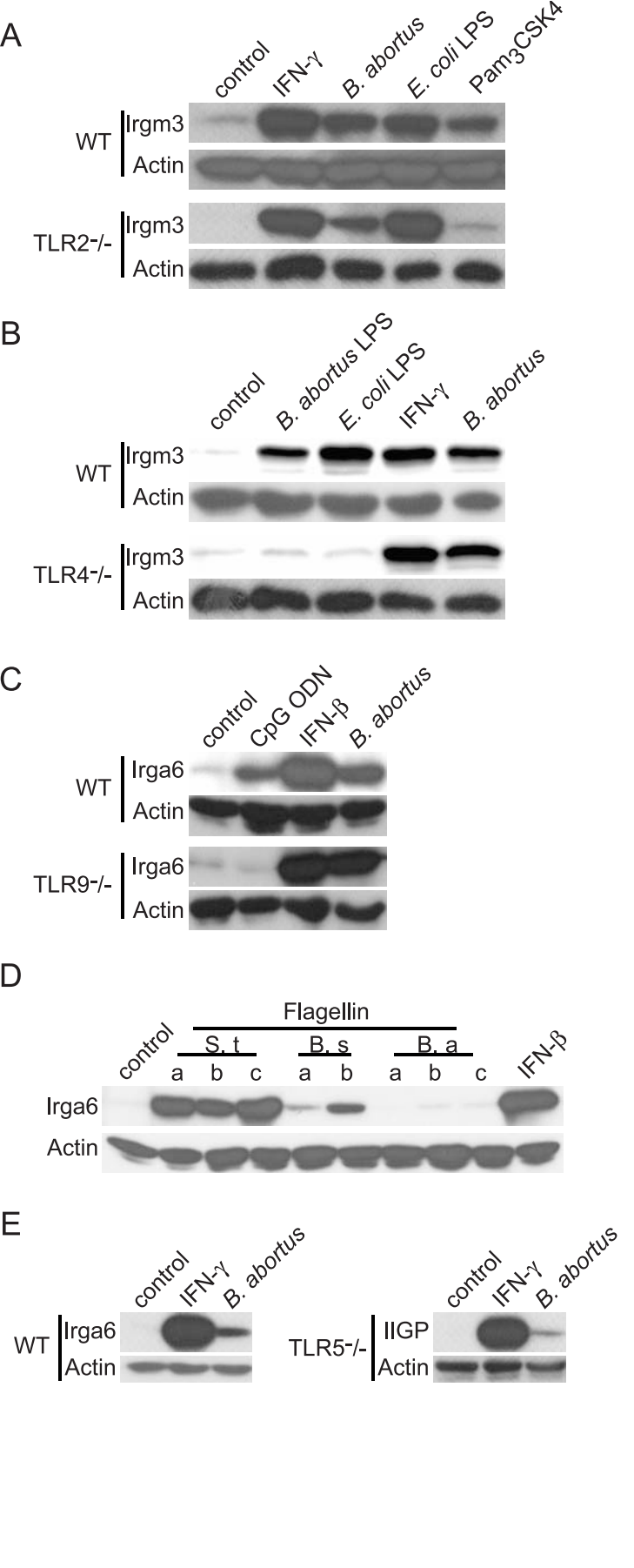
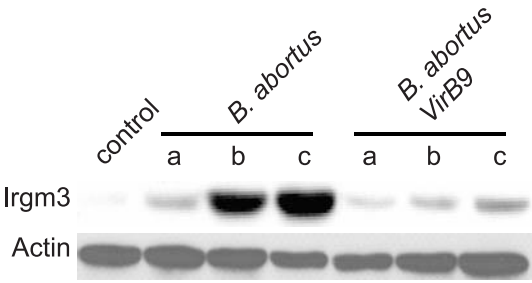

A
control
IFN-γ
B. abortus
E. coli LPS
Pam3CSK4
WT
Irgm3
Actin
TLR2-/-
Irgm3
Actin
B
control
B. abortus LPS
E. coli LPS
IFN-γ
B. abortus
WT
Irgm3
Actin
TLR4-/-
Irgm3
Actin
C
control
CpG ODN
IFN-β
B. abortus
WT
Irga6
Actin
TLR9-/-
Irga6
Actin
D
Flagellin
control
S. t
B. s
B. a
a b c a b a b c
IFN-β
Irga6
Actin
E
control
IFN-γ
B. abortus
WT
Irga6
Actin
control
IFN-γ
B. abortus
TLR5-/-
IIGP
Actin

Fig 3

A


control
B. abortus
B. abortus VirB9
a
b
c
a
b
c
Irgm3
Actin

B

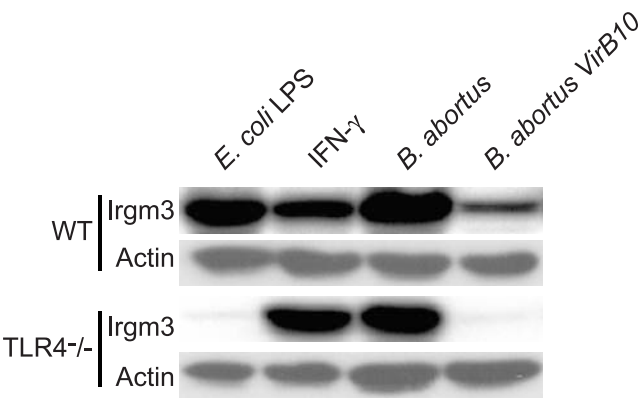

E. coli LPS
IFN-γ
B. abortus
B. abortus VirB10
WT
Irgm3
Actin
TLR4-/-
Irgm3
Actin

Fig 4