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NLRP12 negatively regulates proinflammatory cytokine production and host defense against *Brucella abortus*

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Abstract

Brucella abortus is the causative agent of brucellosis, which causes abortion in domestic animals and undulant fever in humans. This bacterium infects and proliferates mainly in macrophages and dendritic cells where is recognized by pattern recognition receptors (PRRs) including Nod-like receptors (NLRs). Our group recently demonstrated the role of AIM2 and NLRP3 in *Brucella* recognition. Here, we investigated the participation of NLRP12 in innate immune response to *B. abortus*. We found that NLRP12 inhibits the early production of IL-12 in bone marrow-derived macrophages upon *B. abortus* infection. We also observed that NLRP12 suppresses *in vitro* NF-κB and MAPK signaling in response to *Brucella*. Moreover, we showed that NLRP12 modulates caspase-1 activation and IL-1β secretion in *B. abortus* infected-macrophages. Furthermore, we observed that mice lacking NLRP12 were more resistant in the early stages of *B. abortus* infection. NLRP12$^{-/-}$ infected-mice presented reduced bacterial burdens in the spleens and increased production of IFN-γ and IL-1β compared to wild-type controls. In addition, NLRP12 deficiency leads to reduction in granuloma number and size in mouse livers. Altogether, our findings suggest that NLRP12 plays an important role in regulating negatively the early inflammatory responses against *B. abortus*.

Keywords

Innate immunity; Nod-like receptors; inflammasome; NLRP12; *Brucella abortus*

Introduction

Brucella abortus is a Gram-negative facultative intracellular bacterium that induces brucellosis, a major worldwide zoonotic disease. In cattle, *B. abortus* infection leads to infertility and abortion, resulting in considerable economic loss [1]. In humans, *B. abortus*

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Conflict of interest disclosure

The authors have no financial or commercial conflicts of interest.

causes undulant fever, endocarditis, arthritis, osteomyelitis and neurologic disorders [2]. Human brucellosis occurs through inhalation of aerosols containing the pathogen, contact with infected animals, or, more often, consumption of unpasteurized milk or dairy products [3].

In hosts, *B. abortus* survives and replicates predominantly in macrophages and dendritic cells, manipulating host cell vesicular-trafficking pathways and creating a *Brucella*-containing vacuole (BCV). To establish an intracellular replication niche, *Brucella* delivers effector proteins into the host cytosol through a type IV secretion system (T4SS) encoded by the *virB* operon [4]. *Brucella* is recognized by the host using germline-encoded pattern recognition receptors (PRRs) such as TLRs (Toll-like receptors) and NLRs (Nod-like receptors). These sensors trigger the production of pro-inflammatory cytokines leading to the development of a type 1 pattern of immune response that is critical for bacterial clearance and infection control [5]. TLRs are transmembrane receptors, that recognize and bind pathogen-associated molecular patterns (PAMPs), resulting in signal transduction and translocation of NF-κB transcription factor to the nucleus and phosphorylation of mitogen-activated protein (MAP) kinases p38, JNK and ERK [6]. Our laboratory and others have described the involvement of several TLRs and TLRs-associated pathways, such as TLR9 and MyD88, in the recognition of *B. abortus* [7–13].

NLRs are cytoplasmic receptors that sense different PAMPs and DAMPS (danger-associated molecular patterns), and serve as regulators of gene expression by modulating signaling pathways of MAPK and NF-κB as well as participate in the formation of inflammasomes. As cytosolic sensors, NLRs play a critical role in immune response to intracellular pathogens [14]. The NLRs NOD1 and NOD2 recognize bacterial peptidoglycan fragments and recruit the adaptor protein Rip2 to induce a proinflammatory response [15]. Our group demonstrated that bone-marrow-derived macrophages (BMDMs) from NOD1, NOD2, and Rip2 deficient mice possess reduced production of TNF-α compared to wild-type (WT) animals infected with *B. abortus*. However, these proteins had no role in resistance to *Brucella* infection *in vivo* [16]. Some NLRs such as NLRP1, NLRC4, NLRP3, NLRP6, NLRP12 and AIM2 assemble a multimeric complex with the adapter protein ASC and pro-caspase-1 called inflammasome [17, 18]. After being recruited for the inflammasome, caspase-1 is activated, and promotes processing of pro-IL-1β and pro-IL-18 to their mature forms as well as an inflammatory cell death known as pyroptosis [19, 20]. In particular, NLRP3 responds to a variety of pathogens and stimuli [21] and AIM2 recognizes cytoplasmic dsDNA [22, 23]. Our laboratory recently described that both NLRP3 and AIM2 receptors are involved in *in vitro* IL-1β secretion in response to *B. abortus,* and knockout (KO) mice of each receptor are more susceptible to murine brucellosis compared to WT animals [24]. However, it remains unclear if other NLRs are involved in the recognition of *B. abortus*.

NLRP12 (also known as Nalp12, Monarch-1 and Pypaf 7) is a NLR member expressed in immune cells, and its ligand is unknown [25]. NLRP12 was initially described as an activator of caspase-1 and NF-κB signaling in overexpression studies [18]. However, subsequent reports describe NLRP12 as a suppressor of pro-inflammatory signaling and suggest inflammasome-independent functions. NLRP12 has been implicated in

autoinflammatory disorders, colon inflammation and tumorigenesis and host resistance to infectious diseases [25]. Nonetheless, the function of NLRP12 in immune responses against bacterial infections is still not fully addressed.

Herein, we investigate the role of NLRP12 in response to *B. abortus* infection. We observed that NLRP12 inhibits NF-κB and MAPK signaling and caspase-1 activation in BMDMs. Furthermore, in a model of murine brucellosis the absence of NLRP12 conferred host resistance to *Brucella* infection. Collectively, our results suggest an important role of NLRP12 in modulating the early inflammatory responses against *B. abortus*.

Results

NLRP12 negatively regulates IL-12 production in *B. abortus*-infected macrophages

Upon recognition by innate immune receptors, *B. abortus* triggers the production of proinflammatory cytokines such as IL-12 and TNF-α [26]. We first investigated whether NLRP12 participates in the regulation of *in vitro* proinflammatory cytokine production in *B. abortus*-infected BMDMs. After 5 hours of infection, BMDMs from NLRP12$^{-/-}$ mice infected with *B. abortus* S2308 displayed an increased production of IL-12, compared with WT BMDMs (Figure 1A). At the same time, infected NLRP12$^{-/-}$ BMDMs presented no statistically difference in IL-6 and TNF-α production (Figure 1B, 1C), but a slightly elevated IL-6 level was observed relative to WT counterparts. After 24 hours of infection, no difference was observed in cytokine production between infected macrophages from NLRP12$^{-/-}$ and WT mice (Figure 1 D–F), suggesting that NLRP12 plays a role in the early innate immune response against *B. abortus in vitro.* Additionally, we measured the level of *NLRP12* expression by qPCR in unmatured bone marrow cells and BMDM. As shown in Supplementary Figure 1, there is a small reduction in *NLRP12* mRNA levels in BMDM compared to unmatured bone marrow cells; however, *NLRP12* mRNA transcripts were also detected in 10 days matured bone marrow cells (BMDMs).

NLRP12 antagonizes NF-κB and MAPK signaling in response to *B. abortus*

The recognition of *B. abortus* by innate immune receptors results in the activation of different signaling pathways, culminating in the expression of several proinflammatory genes [13, 27]. We next assessed the potential role of NLRP12 in regulating NF-κB and MAPK signaling pathways in BMDMs upon *B. abortus* infection. *B. abortus* induced phosphorylation of p65, JNK, ERK1/2 and p38 in both C57BL/6 and NLRP12$^{-/-}$ macrophages (Figure 2A). However, NLRP12 participates in the regulation of phosphorylation of p65, JNK and p38 in infected-BMDMs, since the lack of NLRP12 leads to increased phosphorylation of those kinases particularly at 4 hours postinfection (Figure 2B). We further investigated if non-canonical NF-κB signaling pathway was altered in NLRP12$^{-/-}$ cells, by examining phosphorylation of p100. We did not detect phosphorylation of this kinase in WT and NLRP12 KO macrophages in response to *B. abortus*. Also, we did not observe any modulation of p38 signaling by NLRP12 in response to LPS from *Escherichia coli*. These data are in agreement with the augmented IL-12 production observed in *B. abortus* NLRP12$^{-/-}$ BMDMs and suggest that NLRP12 dampens cytokine production by inhibiting NF-κB and MAPK phosphorylation.

NLRP12 modulates inflammasome activation in response to *B. abortus*

Our group recently showed that the adaptor protein ASC is essential for caspase-1 activation and IL-1β secretion during *B. abortus* infection. In addition, the *B. abortus* T4SS *virB* is necessary for inflammasome activation [24]. To determine whether NLRP12 has a role in inflammasome activation upon *B. abortus* infection, we infected C57BL/6, NLRP12$^{-/-}$ and caspase-1$^{-/-}$ BMDMs with *B. abortus* S2308 or *virB* mutant for 17 hours. IL-1β secretion induced by *B. abortus* S2308 was significantly increased in NLRP12$^{-/-}$ macrophages compared to WT cells (Figure 3A). Furthermore, we confirmed that *Brucella* T4SS is important for IL-1β release, as we observed a drastic reduction of IL-1β secretion in macrophages infected with *virB* mutant strain. Deficiency in NLRP12 did not affect the production of IL-1β triggered by nigericin, used as positive control. As expected, no IL-1β secretion was detected in caspase-1$^{-/-}$ BMDMs. We further investigated whether NLRP12 modulates inflammasome activation by the cleavage of caspase-1 as determined by immunoblotting of the supernatant from those macrophages. No cleaved caspase-1 was detected from *virB*-infected BMDMs. Also, NLRP12$^{-/-}$ macrophages infected with *B. abortus* S2308 presented more pro-caspase-1 and active caspase-1 compared to WT BMDMs (Figure 3B and 3C). Interestingly, we did not observe modulation of ASC and IL-18 expression by NLRP12 in *B. abortus*-infected BMDMs (Supplementary Figure 2). Taken together these results indicate that in response to *B. abortus*, NLRP12 modulates *in vitro* pro-caspase-1 expression leading to reduced caspase-1 level and IL-1β secretion.

NLRP12 deficiency protects mice from *B. abortus* infection

To determine the role of NLRP12 *in vivo* following *B. abortus* infection, C57BL/6 and NLRP12$^{-/-}$ mice were infected intraperitoneally with virulent *B. abortus* S2308. After 72 hours of infection, the bacterial burden from spleens was determined. NLRP12$^{-/-}$ mice were significantly more resistant to *B. abortus* infection and presented a reduced bacterial load compared to WT controls (Figure 4A). Lack of NLRP12 also enhanced host resistance to *B. abortus* at one week postinfection. Conversely, no differences were observed between both mouse groups after two weeks postinfection. Taken together, these results strongly suggest an important early role of NLRP12 in modulating host susceptibility to *B. abortus in vivo*.

Previously, *in vivo* protection against *B. abortus* infection was shown to require the induction of a Th1-type immune response, where IFN-γ is a pivotal cytokine for host control of brucellosis [28, 29]. Thus, to investigate the role of NLRP12 in regulating *in vivo* Th1 response upon *B. abortus* infection, IFN-γ levels in sera of infected mice were evaluated. After 24 hours of infection, NLRP12$^{-/-}$ mice displayed increased systemic production of IFN-γ compared to WT controls (Figure 4B). After 72 hours of infection, augmented production of IFN-γ persisted in mice lacking NLRP12. At this same time interval (72 hrs), we also detected modest but elevated levels of IL-1β in NLRP12$^{-/-}$ mice sera compared to C57BL/6 (Figure 4C). To further investigate the contribution of NLRP12 in host susceptibility against *B. abortus*, we infected WT, NLRP12$^{-/-}$ and IFN-γ$^{-/-}$ mice and monitored survival. IFN-γ$^{-/-}$ mice were used as positive control, due to their enhanced susceptibility to brucellosis. Following infection, all IFN-γ-deficient mice succumbed within 29 days of infection, whereas no mortality was observed in C57BL/6 and NLRP12$^{-/-}$ mice (Figure 4D).

NLRP12 regulates liver granuloma during *B. abortus* infection

Infection with *B. abortus* results in the formation of liver and spleen granulomas, where inflammatory cells aggregate to restrain bacterial growth. After 1 week of infection, granulomas in the liver are conspicuous [30]. At this time, NLRP12$^{-/-}$ mice displayed a significant reduction in granuloma number and size when compared to WT counterparts (Figure 5 A–C). These data suggest that NLRP12 also modulates host liver pathology in the early stages of *Brucella* infection.

Discussion

The innate immune response against *B. abortus* begins with the recognition of bacterial components by PRRs such as TLRs and NLRs. Several studies have explored the involvement of TLRs and their signaling pathways in response to *Brucella*; however, the participation of NLRs in brucellosis is not fully understood [27]. Recently, our group demonstrated the importance of NLRP3 and AIM2 in host susceptibility against *B. abortus* [24]. In this study, we described an anti-inflammatory role of NLRP12 in response to *B. abortus* infection.

B. abortus triggers antigen-presenting cells to produce several proinflammatory cytokines such as TNF-α, IL-6, IL-12, IL-1β and type I IFNs. IL-12 is an important cytokine that drives Th0 cells to differentiate into Th1 effector cells that secrete IFN-γ, a cytokine essential to control *Brucella* infection [31]. In our *in vitro* experiments with BMDMs, we observed that NLRP12 is a negative regulator of IL-12 production upon *B. abortus* infection. Interestingly, we found that NLRP12 attenuates IL-12 secretion at 5 hours but not at 24 hours after infection. Also using BMDMs, Zaki and colleagues demonstrated an early negative regulation promoted by NLRP12 in proinflammatory cytokine production in *Salmonella typhimurium* infection [32]. Another report using NLRP12$^{-/-}$ BMDCs described the role of this receptor in attenuating early cytokine production in response to PAMPs associated with *Escherichia coli*, *Klebsiella pneumoniae* and *Mycobacterium tuberculosis* [33]. Altogether, these findings suggest that NLRP12 plays an important role as an anti-inflammatory mediator in the early innate immune response against different bacterial pathogens.

Multiple signaling pathways lead to proinflammatory cytokine production in response to *B. abortus* [13]. Here, we showed that NLRP12 modulates phosphorylation of NF-κB and MAPK components in BMDMs infected with *B. abortus*. We hypothesized that NLRP12 dampens IL-12 production by modulating these transduction signaling pathways (Figure 6). Potentially, the negative regulation in signaling mediated by NLRP12 also interferes with the expression of other essential inflammatory molecules. In our model of infection, we did not detect activation of the non-canonical (alternative) NF-κB pathway in response to *B. abortus*. However, *Brucella* may activate this alternative pathway in other cell types than macrophages and during longer infection kinetics. In *S. typhimurium*-infected BMDMs, NLRP12 regulates ERK phosphorylation and activation of canonical NF-κB signaling, with no role in the non-canonical pathway [32]. NLRP12 has been implicated to regulate both canonical and alternative NF-κB signaling. However, the regulatory role of NLRP12 in non-canonical NF-κB has been described mainly in biochemical assays, dendritic cells and colon

cancer models [34, 35]. Due to the slow kinetics and dependence of *de novo* protein synthesis for the alternative NF-κB pathway [36], we speculate that canonical signaling plays a much important role in the early response to bacterial infections in murine macrophages.

Inflammasomes play a central role in host defense against pathogens and endogenous danger signals. Inflammasome formation leads to caspase-1 activation and IL-1β maturation, contributing to a robust proinflammatory response. During *B. abortus* infection, ASC inflammasome is indispensable for inducing the activation of caspase-1 and secretion of IL-1β, whereas NLRP3 and AIM2 are partially required for IL-1β maturation [24]. In this study, we observed that mature IL-1β, pro-caspase-1 and active caspase-1 were markedly increased in *B. abortus*-infected NLRP12$^{-/-}$ BMDMs. Our data demonstrates that NLRP12 does not affect the expression of ASC and pro-IL-18 (Figure S2). Therefore, we hypothesize that NLRP12 acts interfering in pro-caspase-1 expression and caspase-1 cleavage in response to *Brucella* probably inhibiting signal 1 via NF-κB (Figure 6). Further experiments are required to elucidate the underlying mechanisms by which NLRP12 modulates expression of some inflammasome components and IL-1β secretion following *B. abortus* infection. In contrast to our findings, other studies reported that NLRP12 does not contribute to IL-1β maturation in response to derived-microbial PAMPs or *S. typhimurium* [32]. Another study determined that NLRP12 is an inflammasome component involved in the recognition of *Yersinia pestis* and positively regulates IL-1β and IL-18 production [37]. To the best of our knowledge, this is the first study to describe a negative regulation of caspase-1 activation mediated by NLRP12 in response to a bacterial infection. NLRP12-mediated suppression of proinflammatory signaling was also shown to play a central role in the attenuation of colon inflammation and tumorigenesis in mice [35, 38]. More recently, an unexpected role for NLRP12 was defined as intrinsic negative regulator of pathogenic T cell responses in autoinflammatory disease [39]. In this model, dysregulated production of IL-4 promoted atypical neuroinflammatory disease in NLRP12$^{-/-}$ mice. Overall, the function of NLRP12 appears to be dependent on the type of cells, pathogens, and stimuli analyzed.

The protective host response against *B. abortus* requires Th1-type cytokines such as IFN-γ that activates macrophage microbicidal mechanisms. In fact, IFN-γ$^{-/-}$ mice fail to control *Brucella* replication and succumb to infection [28]. In the present study, absence of NLRP12 leads to protection against *B. abortus* infection *in vivo*. Mice deficient for NLRP12 had reduced bacterial counts in the spleen and higher levels of serum IFN-γ at 72 hours after infection. Moreover, a greater reduction in granuloma number and size was detected in NLRP12$^{-/-}$ mice at 1 week of infection. The formation of granulomas is an important component of coordinated antibacterial defenses, in which lymphocytes cooperate with macrophages to restrain bacterial growth. Previous studies have described hepatic microgranulomas during systemic infections with pathogenic *Brucella* spp. in the mouse [40]. The granulomas induced by *Brucella* are mainly composed of CD11b^{+} F4/80^{+} MHC-II^{+} cells and a fraction of these cells also expressed CD11c marker and appeared similar to inflammatory DCs [41]. Since, NLRP12 is important in maintaining neuthophils and DCs in a migration-competent state [42] and this sensor also affected macrophages content in BALF from *Klebsiella penumoniae* infected mice [33], we hypothesize that lack of NLRP12 in *Brucella* infected animals might have influenced DCs and macrophages migration during

granuloma formation. These data are consistent with our *in vitro* findings that demonstrate the negative regulation of immune response promoted by NLRP12. Conversely, mice lacking ASC, caspase-1, AIM2, and NLRP3 are more susceptible to *B. abortus* [24]. Because the present *in vivo* data indicate that NLRP12 has a role in attenuating inflammation at the early stages of *Brucella* infection, we hypothesize that NLRP12 likely acts upstream of these inflammasome components.

In summary, our findings demonstrated that NLRP12 is a negative regulator of proinflammatory response against *B. abortus*. NLRP12 inhibits *in vitro* production of IL-12, modulates expression of some inflammasome components and IL-1β secretion upon *B. abortus* infection. NLRP12 also plays a role *in vivo*, attenuating IFN-γ response and contributing to host susceptibility in the early immune response to murine brucellosis. Nevertheless, the *B. abortus* ligands required for NLRP12 recognition remain to be identified.

Material and methods

Mice

Wild-type C57BL/6 (WT) mice were purchased from the Federal University of Minas Gerais (UFMG), NLRP12$^{-/-}$, caspase-1$^{-/-}$ and IFN-γ$^{-/-}$ were described previously [29, 43, 44]. Mice 6 to 8 week of age were used for *in vivo* experiments and/or to obtain macrophages from bone marrow cells. Food and water were provided *ad libitum* and all procedures performed in this study were approved by the local ethical committee (CETEA # 128/2014).

Bacteria

Bacteria used included *B. abortus* strain (S) 2308 obtained from our laboratory collection and the *B. abortus virB* operon mutant strain kindly provided by Dr. Renato de Lima Santos (UFMG). Bacteria were grown in BB liquid medium (Difco, Detroit, MI, USA) at 37°C under constant agitation for 72 hours. Bacterial cultures were pelleted and suspended in phosphate-buffered saline (PBS) containing 25% of glycerol, and then aliquoted and stored at −80 C until use. Aliquots were serially diluted and plated in BB medium containing 1.5 % bacteriological agar (BB agar). After incubation for 72 hours at 37°C, bacterial concentration was determined by counting CFUs.

Bacterial counting in *B. abortus* infected mice

Five mice from each group (C57BL/6 or NLRP12$^{-/-}$) were infected intraperitoneally (i.p.) with 1×10^6 virulent *B. abortus* S2308 in 100μl of PBS. After 72h, 1 and 2 weeks postinfection, mice were sacrificed and spleens were used to determine the number of bacteria by CFU counting. Spleens harvested from each animal were weighed and macerated in 10mL of saline (NaCl 0.9%). To determine bacterial burden, spleens were serially diluted in saline and plated in duplicate on BB agar. Plates were incubated for 3 days at 37°C and CFU number was determined.

Survival analysis of *B. abortus* infected mice

Eight mice from each group (C57BL/6, NLRP12$^{-/-}$ and IFN-γ$^{-/-}$) were infected i.p with 1×10^6 virulent *B. abortus* S2308 as described above and were monitored daily for 60 days.

Bone marrow-derived macrophages (BMDMs)

Bone marrow cells were flushed from femurs and tibias of C57BL/6, NLRP12$^{-/-}$ or caspase-1$^{-/-}$ mice and were differentiated into macrophages as previously described [24]. Briefly, cells were seeded in 24-well plates (5×10^5 cells/well) and cultured in DMEM (Gibco, Carlsbad, CA, USA) containing 10% L929 cell-conditioned medium (LCCM), 10% FBS (HyClone, Logan, UT, USA), 1% penicillin-streptomycin and 1% HEPES, at 37°C in an atmosphere of 5% CO_2. At day 4 of differentiation, LCCM was added (100uL/well) and at day 7, culture medium was replaced with fresh medium containing 10% LCCM. At day 10, cells were completely differentiated into macrophages and culture medium was replaced with antibiotic-free DMEM plus 1% FBS. NLRP12 expression in unmatured bone marrow cells and macrophages was evaluated by Real-time PCR (Supplementary Figure 1).

Real-time PCR (RT-PCR)

BMDMs from C57BL/6 and NLRP12$^{-/-}$mice were infected with *B. abortus* S2308 (multiplicity of infection [MOI] 100:1) for 24 hours and total RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized by reverse transcription (RT) from 1μg of total RNA, and was used to perform RT-PCR in a final volume of 10μl containing SYBR green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) and 20μM of primers. RT-PCR was performed in triplicate, on an ABI 7900 Real-time PCR system (Applied Biosystems). The primers used for the NLRP12 [32], ASC [45] and IL-18 [46] and β-actin [12] genes were as follows: NLRP12 forward, 5′-CCTCTTTGAGCCAGACGAAG-3′; NLRP12 reverse, 5'-GCCCAGTCCAACATCACTTT-3'; ASC forward, 5'-CAGAGTACAGCCAGAACAGGACAC-3': ASC reverse, 5'-GTGGTCTCGCACGAACTGCCTG-3': IL-18 forward, 5'-GCCTCAAACCTTCCAAATCA-3': IL-18 reverse, 5'- TGGATCCATTTCCTCAAAGG-3': β-actin forward, 5'-AGGTGTGCACCTTTTATTGGTCTCAA-3'; and β-actin reverse, 5'-TGTATGAAGGTTTGGTCTCCCT-3'. The levels of mRNAs are presented as relative expression units after normalization to the β-actin gene.

Cytokines measurement

To assay *in vitro* production of IL-12, IL-6 and TNF-α, BMDMs were infected with *Brucella abortus* S2308 (MOI 100:1) for 5 or 24 hours and supernatants were harvested. For *in vivo* determination of IFN-γ and IL-1β levels, C57BL/6 or NLRP12$^{-/-}$ mice were infected i.p. with 1×10^9 virulent *B. abortus* S2308 and sacrificed at the indicated times. Blood was collected and purified sera were used for cytokine analysis. All cytokines were measured using commercially available ELISA Duoset kits (R&D Systems, Minneapolis, MN, USA).

Western blotting for cell signaling events

To detect phosphorylation of MAPK and NF-κB, BMDMs were serum starved for 16 hours and infected with *B. abortus* S2308 (MOI 1000:1) or stimulated with *E.coli* LPS (1μg/ml) for 30 min. At the indicated times, cells were lysed with M-PER™ Mammalian Protein Extraction Reagent (Thermo Fisher Scientific) supplemented with protease and phosphatase inhibitors (Roche). Protein concentration was determined using Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific). Equal amounts of proteins were separated on 15% SDS-PAGE gels and transferred to nitrocellulose membranes (Amersham Biosciences, Uppsala, Sweden) in transfer buffer (50mM Tris, 40mM glycine, 10% methanol). Membranes were blocked for 1 hour in TBS with 0.1% Tween-20 containing 5% nonfat dry milk and incubated overnight with primary antibodies (ERK1/2, p38, JNK, p65, phospho-ERK1/2, phospho-p38, phospho-JNK, phospho-p65, phospho-p100, and β-actin [Cell Signaling Technology, Danvers, MA, USA]) at 4°C. Membranes were incubated with horseradish peroxidase-conjugated secondary antibody and Luminol chemiluminescent HRP substrate (Millipore, Billerica, MA, USA) was used for antibody detection. Densitometry analysis was performed using ImageQuant TL Software (GE Healthcare, Buckinghamshire, United Kingdom), and band intensities were normalized to total proteins or β-actin. Data were obtained relative to the level of C57BL/6 BMDMs infected with *B. abortus* for 30 min assigned arbitrarily with the value of 1.0.

Detection of activated caspase-1 and secreted IL-1β

BMDMs were infected with *Brucella abortus* S2308 or *virB* mutant (MOI 100:1) for 17 hours. As a positive control, cells were primed with 1 μg/ml of *E. coli* LPS (Sigma-Aldrich, St. Louis, MO, USA) for 4h and stimulated with 20μM nigericin sodium salt (Sigma-Aldrich) for 30 minutes. Culture supernatants were collected and cells were lysed with M-PER™ Mammalian Protein Extraction Reagent (Thermo Fisher Scientific) for western blotting analysis as described above. Processed p20 subunit of caspase-1 (caspase-1 p20) and unprocessed caspase-1 (pro-caspase-1) were detected using primary antibody anti-caspase-1 (p20) (Adipogen, San Diego, CA, USA). Densitometry analysis was performed using ImageQuant TL Software (GE Healthcare). Band intensities were normalized to the level of pro-caspase-1 related to C57BL/6 BMDMs infected with *B. abortus*. IL-1β was measured from culture supernatants using IL-1β ELISA Duoset kit (R&D Systems) according to the manufacturer's instructions.

Histopathology

Five mice (C57BL/6 or NLRP12$^{-/-}$) from each group were infected i.p. as described above. Liver medial lobes from 1 week *B. abortus*-infected mice were fixed in 10% buffered formaldehyde solution and embedded in paraffin by standard techniques. Histological sections (5μM thick) were stained with hematoxylin and eosin (HE). Total number of granulomas was unbiasedly determined using an Olympus CX31 microscope with a 20× objective. Digital images of 15 granulomas/animal were acquired using an Olympus SC30 camera. The area of histological sections and the size of granulomas were calculated using the Image Tool 3.0 software, and total numbers of granulomas were normalized for a 50mm^2 tissue area.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software, version 5 (GraphPad Software, San Diego, CA, USA). Data were analyzed using Two-way ANOVA or Student's *t* test to calculate the significance differences. Data are presented as mean ± SEM, and a value of $P \leq 0.05$ was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BMDMs	bone-marrow-derived macrophages
LCCM	L929 cell-conditioned medium
NLRs	Nod-like receptors
MOI	multiplicity of infection
PAMPs	pathogen-associated molecular patterns
PRRs	pattern recognition receptors
T4SS	type IV secretion system
TLRs	Toll-like receptors
WT	wild-type

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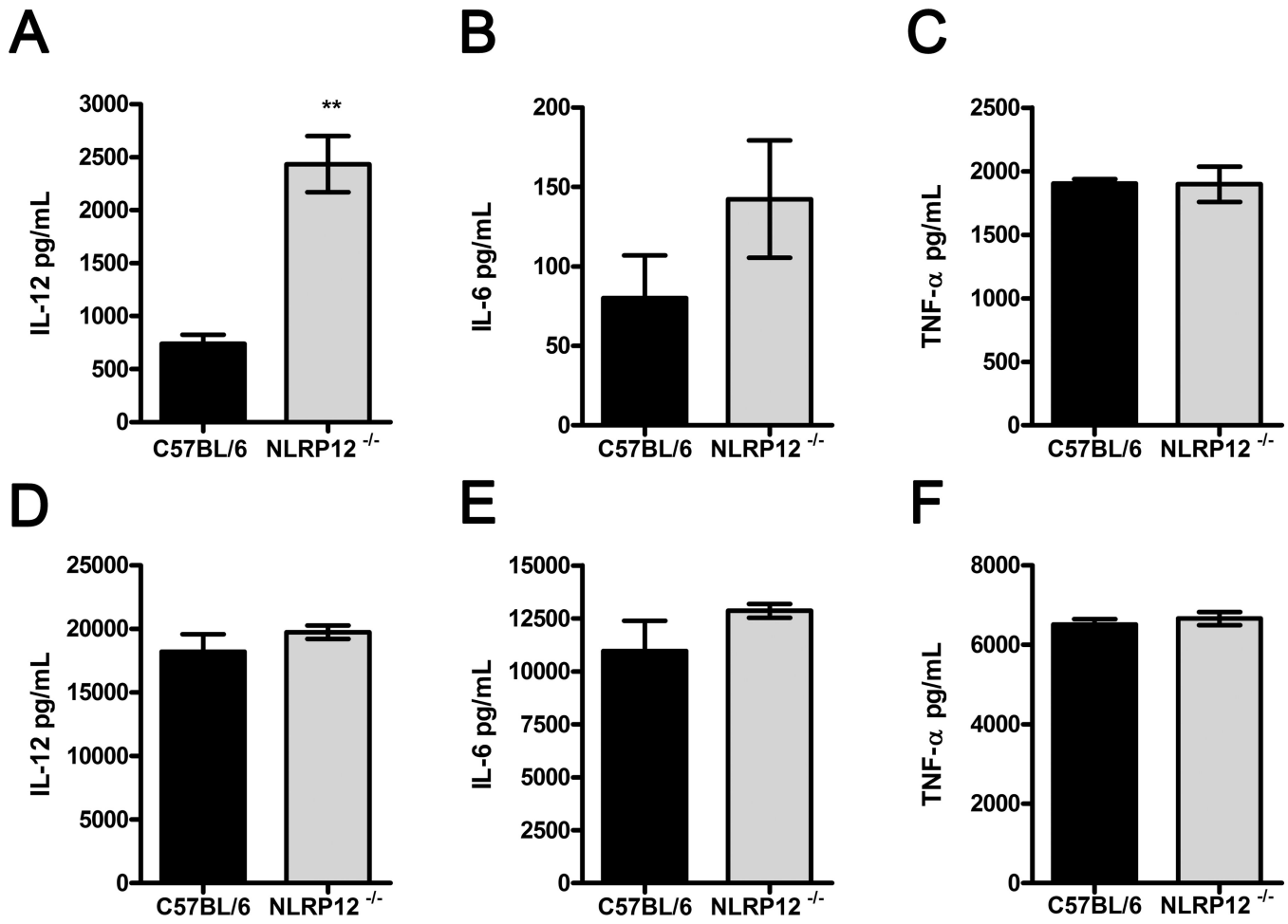

Figure 1.

NLRP12 dampens IL-12 production in *B. abortus*-infected macrophages. BMDMs from C57BL/6 and NLRP12$^{-/-}$ mice were infected with *B. abortus* S2308 at a MOI of 100:1. After five (A–C) and 24 hours (D–F) of infection culture supernatants were analyzed for IL-12, IL-6 and TNF-α production by ELISA. Data are expressed as mean ± SEM of three samples per group analyzed in triplicate from one experiment representative of four independent experiments. ** $P \leq 0.005$ in comparison to C57BL/6 BMDMs using Student's *t*-test.

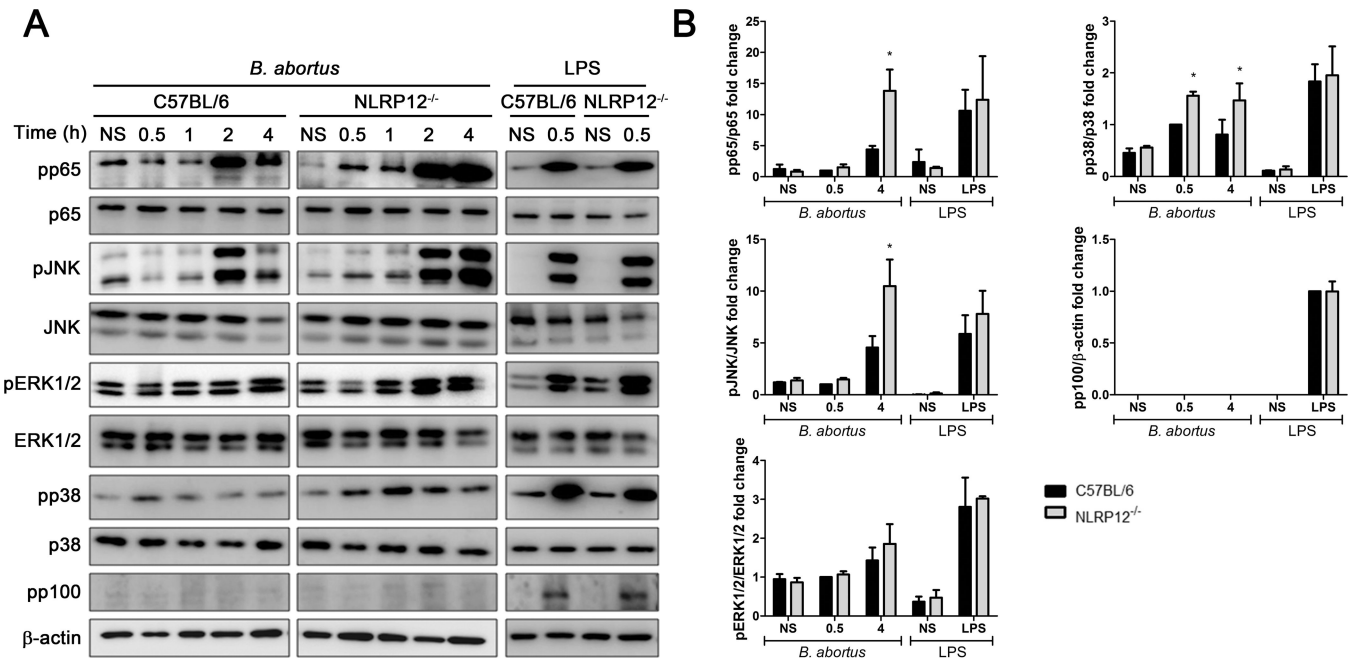

Figure 2.

NLRP12 negatively regulates NF- κB and MAPK signaling in response to *B. abortus*. BMDMs from C57BL/6 and NLRP12$^{-/-}$ mice were non-stimulated (NS) or infected with *B. abortus* S2308 at a MOI of 1000:1 for the indicated times or stimulated with LPS (1μg/ml) for 30 min and lysed for western blot analysis. (A) Cell lysates were then separated by SDS-PAGE, blotted, and probed with the indicated antibodies. β-actin was used as loading control. (B) Bar graphs show densitometry analysis of pp65, pJNK, pERK1/2 and pp38 relative to their respective total proteins or pp100 relative to β-actin. Data shown in all panels are expressed as mean ± SEM of three independent experiments. * $P \leq 0.05$ in comparison to C57BL/6 BMDMs using Two-way ANOVA.

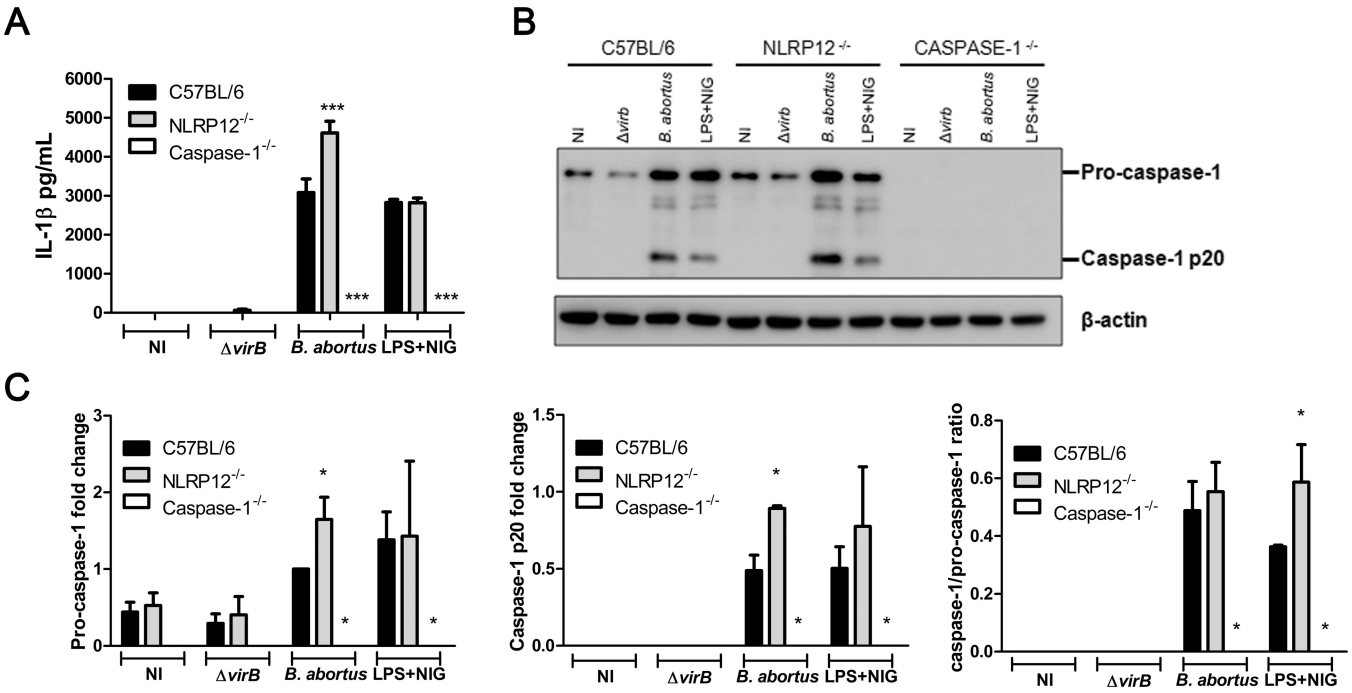

Figure 3.

NLRP12 inhibits *B. abortus*-induced IL-1β secretion and caspase-1 activation. BMDMs from C57BL/6, NLRP12$^{-/-}$ and caspase-1$^{-/-}$ mice were non-infected (NI) or infected with *B. abortus* S2308 or Δ*virB* at a MOI of 100:1 for 17 hours or stimulated with LPS and nigericin. (A) IL-1β levels in culture supernatants were quantified by ELISA. Data are shown as mean ± SEM of three samples per group analyzed in triplicate. *** $P \leq 0.001$ between knockout BMDMs and C57BL/6 BMDMs, Two-way ANOVA. (B) Culture supernatants were separated by SDS-PAGE, blotted, and probed with anti-caspase-1. Detection of β-actin in cell lysates was used as loading control. (C) Bar graphs show densitometry analysis of pro-caspase-1, caspase-1 p20 and caspase-1/pro-caspase-1 ratio. Data shown in all panels are expressed as mean ± SEM of three independent experiments. * $P \leq 0.05$ in comparison to C57BL/6 BMDMs using Two-way ANOVA.

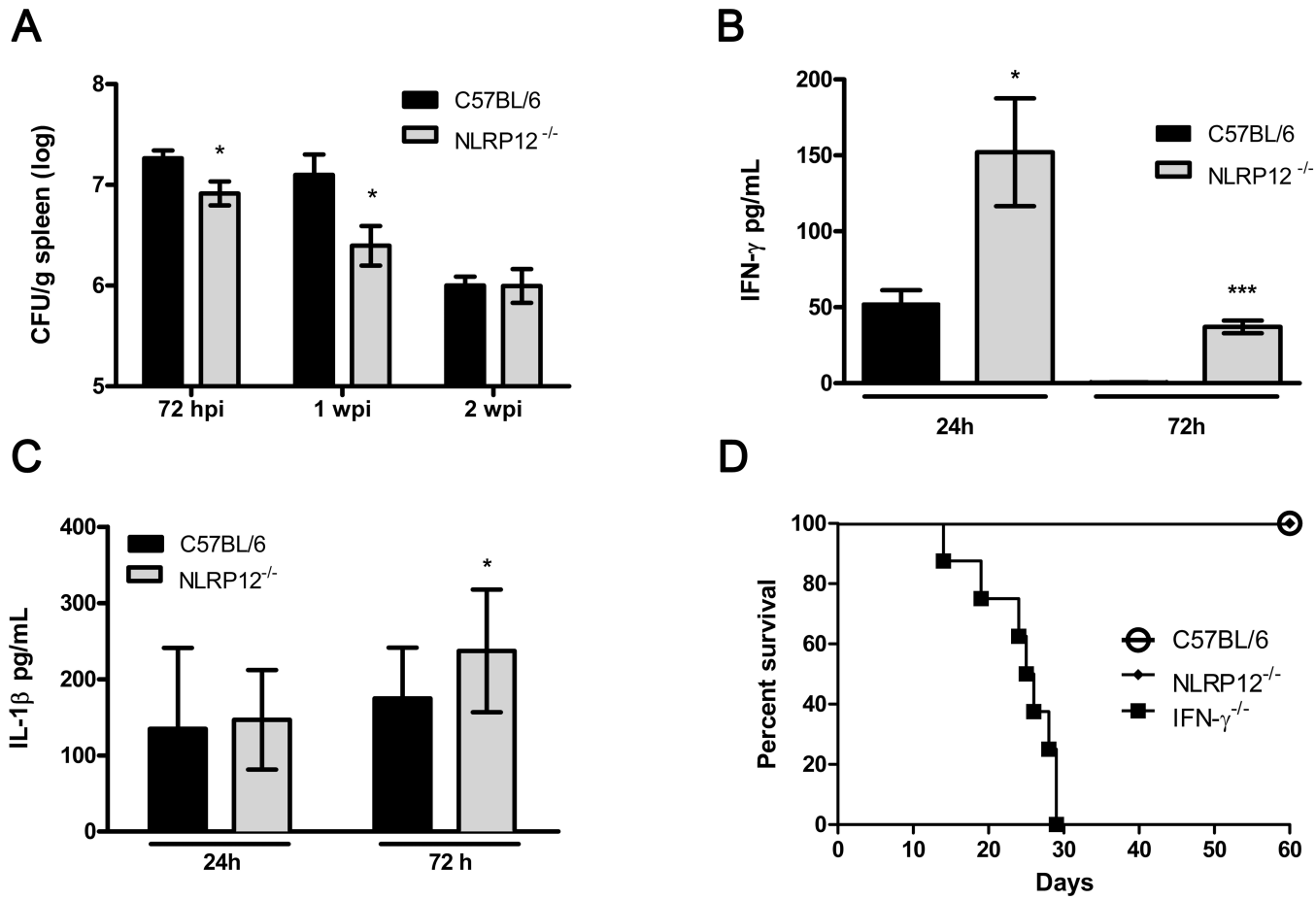

Figure 4.

NLRP12 deficiency partially protects mice from *B. abortus* infection. C57BL/6 (n=5) and NLRP12$^{-/-}$ (n=5) mice were infected i.p. with (A) 1×10^6 units of *B. abortus* S2308, and at 72 hours (hpi), 1 or 2 weeks postinfection (wpi) bacterial loads in the spleens were determined and expressed as log colony forming units (CFU); or with (B,C) 1×10^9 *B. abortus* S2308 and sera were collected at indicated times to determine (B) IFN-γ and (C) IL-1β levels by ELISA. (D) C57BL/6 (n=8), NLRP12$^{-/-}$ (n=8) and IFN-γ$^{-/-}$ (n=8) mice were infected i.p with 1×10^6 *B. abortus* S2308. Survival of the mice was monitored daily. All data are the mean ± SEM. Data are representative of two independent experiments. * $P \leq 0.05$ and *** $P \leq 0.0001$ compared to C57BL/6, Student's *t*-test.

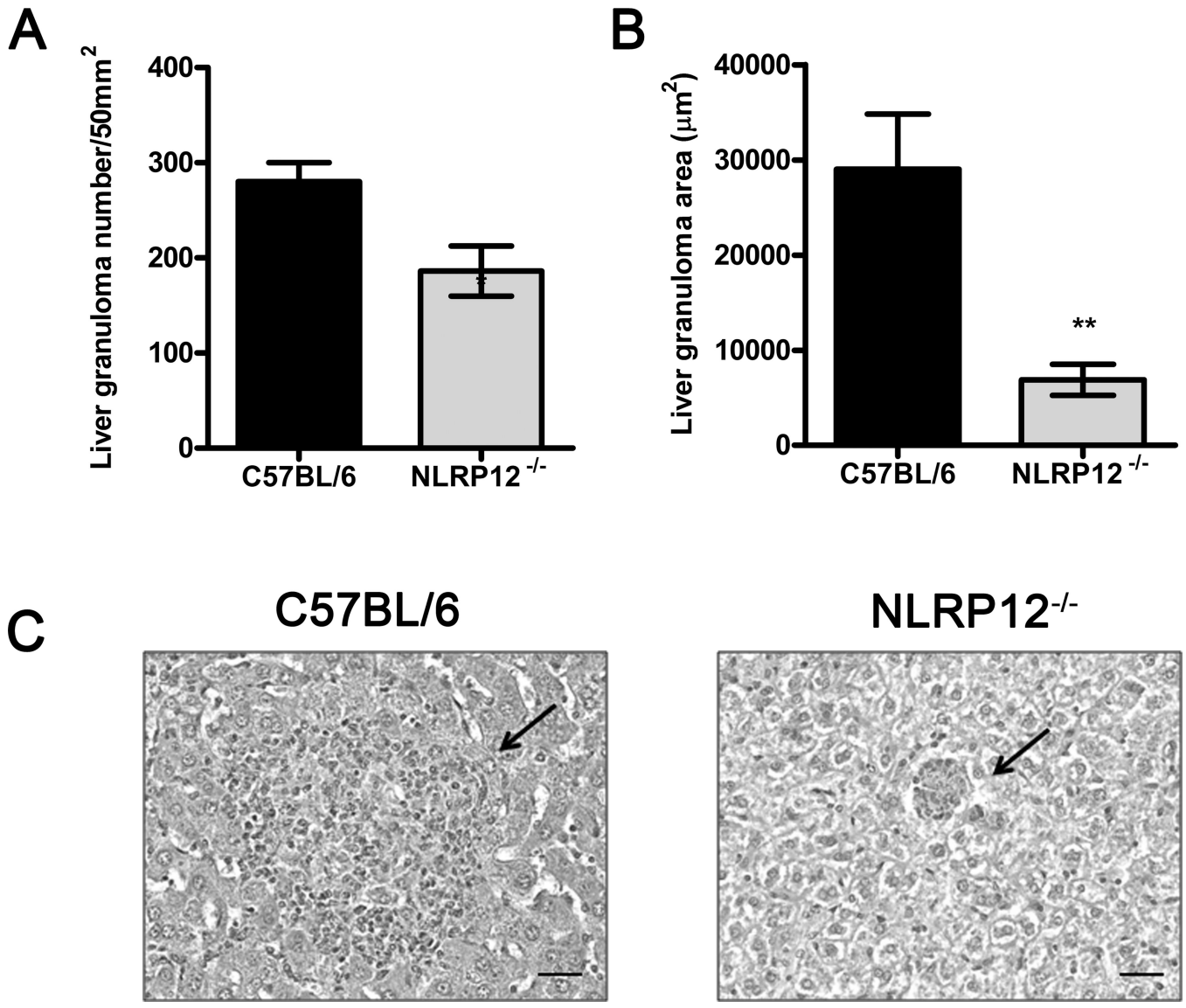

Figure 5.

NLRP12 modulates granuloma formation during early *B. abortus* infection. C57BL/6 (n=5) and NLRP12$^{-/-}$ (n=5) mice were infected i.p. with 1×10^6 *B. abortus* S2308 for 1 week. Livers were paraffin imbedded for histological analysis and (A) granuloma numbers (B) and surface area of granulomas were determined (μm^2). Bars show mean ± SEM. Data are representative of two independent experiments. * $P \leq 0.05$ and **, $P \leq 0.005$ compared to C57BL/6, Student's *t*-test. (C) Representative H&E staining of hepatic tissue of infected mice. Arrows indicate granulomas in the liver of C57BL/6 (left) and NLRP12$^{-/-}$ (right) mice. Digital images were captured using 20× magnification. Scale bars, 20 μm.

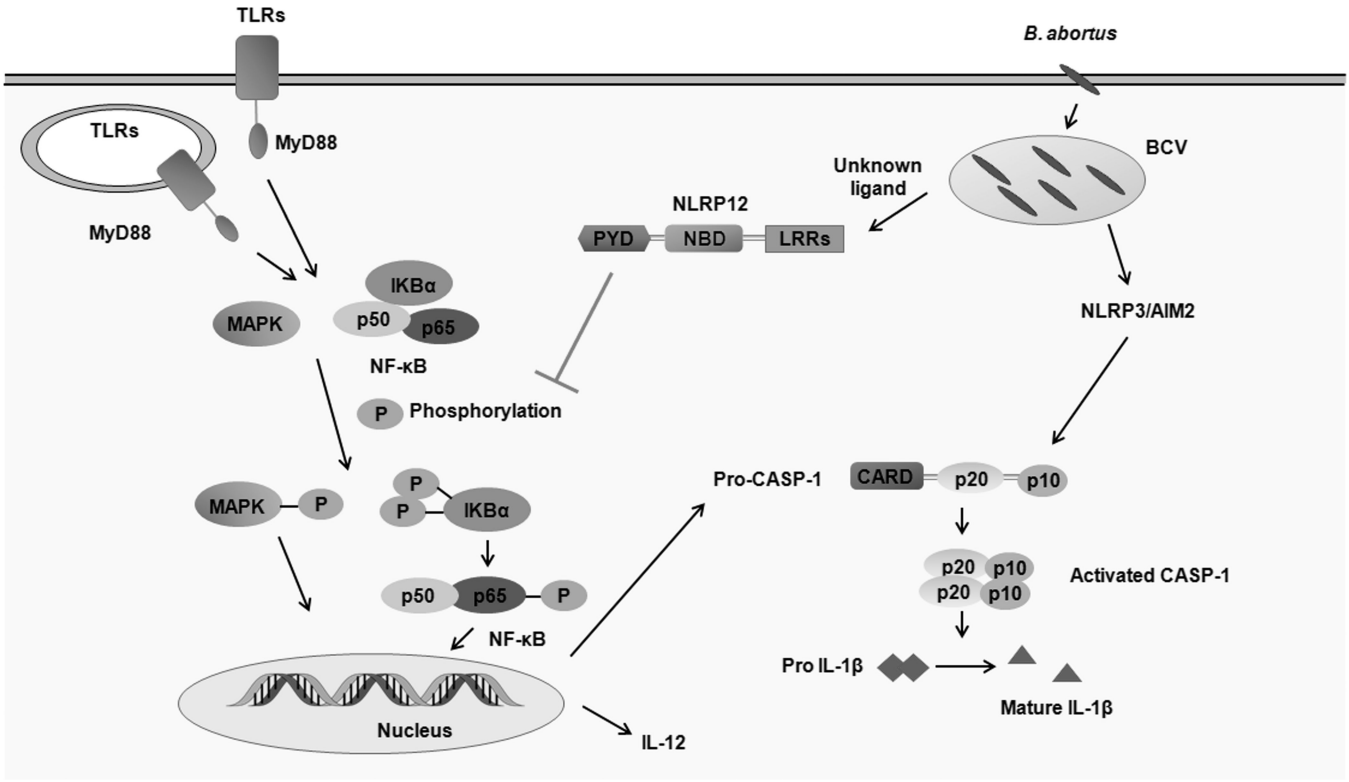

Figure 6.

Model of NLRP12-mediated modulation of proinflammatory response during *B. abortus* infection in murine macrophages. Upon recognition of *B. abortus* components, TLRs via MyD88 trigger activation of NF-κB and MAPK signaling. NLRP12 inhibits phosphorylation of MAPK and the p65 subunit of NF-κB and IL-12 secretion. NLRP12 also modulates pro-caspase-1 expression and mature IL-1β secretion. At present, the NLRP12 agonist is unknown, and it is unclear whether NLRP12 cooperates with other NLRs members to regulate innate immune response. TLRs, Toll-like receptors; MyD88, Myeloid differentiation primary response gene 88; LRRs, leucine-rich repeats; NBD, nucleotide-binding domain; CARD, caspase recruitment domain; PYD, pyrin domain; BCV, *Brucella*-containing vacuole; Casp-1, Caspase-1.