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Central Role of MyD88-Dependent Dendritic Cell Maturation and Proinflammatory Cytokine Production to Control *Brucella abortus* Infection

Gilson Costa Macedo,²* Diogo Matos Magnani,²* Natalia Barbosa Carvalho,* Oscar Bruna-Romero,† Ricardo T. Gazzinelli,* and Sergio Costa Oliveira³*

*Brucella abortus* is a facultative intracellular bacterium that infects humans and domestic animals. The enhanced susceptibility to virulent *B. abortus* observed in MyD88 knockout (KO) mice led us to investigate the mechanisms involved in MyD88-dependent immune responses. First, we defined the role of MyD88 in dendritic cell (DC) maturation. In vitro as well as in vivo, *B. abortus*-exposed MyD88 KO DCs displayed a significant impairment on maturation as observed by expression of CD40, CD86, and MHC class II on CD11c⁺ cells. In addition, IL-12 and TNF-α production was totally abrogated in MyD88 KO DCs and macrophages. Furthermore, *B. abortus*-induced IL-12 production was found to be dependent on TLR2 in DC, but independent on TLR2 and TLR4 in macrophages. Additionally, we investigated the role of exogenous IL-12 and TNF-α administration on MyD88 KO control of *B. abortus* infection. Importantly, IL-12, but not TNF-α, was able to partially rescue host susceptibility in MyD88 KO infected animals. Furthermore, we demonstrated the role played by TLR9 during virulent *B. abortus* infection. TLR9 KO-infected mice showed 1 log *Brucella* CFU higher than wild-type mice. Macrophages and DC from TLR9 KO mice showed reduced IL-12 and unaltered TNF-α production when these cells were stimulated with *Brucella*. Together, these results suggest that susceptibility of MyD88 KO mice to *B. abortus* is due to impaired DC maturation and lack of IL-12 synthesis. Additionally, DC activation during *Brucella* infection plays an important regulatory role by stimulating and programming T cells to produce IFN-γ. *The Journal of Immunology*, 2008, 180: 1080–1087.

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*Brucella* is a facultative intracellular bacterium that infects humans and domestic animals (1). After entering the host, *Brucella* is taken up by dendritic cells (DC)² and macrophages (2). The recognition of microbes by cells of innate immunity involves host cell membrane receptors termed TLR (3). TLRs, upon activation by microbial products, transduce signals via a common adaptor molecule, MyD88, consequently leading to host cell activation (4). MyD88 is critical for TLR-mediated activation of the transcription factor NF-kB and the induction of proinflammatory cytokines (5, 6). Although the available evidence suggests that multiple rather than single TLRs are required for innate defense against most pathogens, it is not clear how signals from different TLRs are orchestrated in generating protective responses against microbial pathogens (7, 8).

In previous studies, we and others showed that *Brucella abortus* signals through TLR2 and TLR4 (9–11) but TLR2 plays no role in controlling the infection. Additionally, Huang et al. (12) demonstrated that heat-killed *B. abortus* (HKBA) activates DC and macrophages to secrete TNF-α and IL-12 by two distinct TLR pathways. TNF-α was TLR2 dependent whereas IL-12 secretion was TLR2 independent but MyD88 dependent. Furthermore, the same group showed that IL-12 synthesis by DC is dependent on TLR9 and that TLR9 knockout (KO) mice failed to mount an effective Th1 response (13). Regarding in vivo infection, Weiss et al. (14) demonstrated that MyD88 is a critical molecule during *B. abortus* infection because MyD88 KO mice did not control the bacteria as efficiently as wild-type, TLR4 KO, TLR2 KO, or TLR2/4 KO mice. However, the mechanisms involved in MyD88-deficient mice susceptibility to brucellosis are not known.

In this study, we confirmed that the MyD88 molecule plays a critical role in host control of *Brucella* infection in vivo, and we defined the immune mechanisms related to this enhanced susceptibility. First, bone marrow (BM)-derived and splenic DCs from MyD88 KO mice do not mature as efficiently as cells from wild-type mice when stimulated with HKBA. Second, DCs and macrophages from MyD88 KO mice do not produce significant amounts of TNF-α and IL-12 compared with wild-type mice. Furthermore, IL-12 was shown to be critical to MyD88 KO resistance because *Brucella* CFU were reduced in spleens of MyD88 KO mice treated with rIL-12. Additionally, MyD88 KO macrophages produce low levels of the inflammatory chemokines RANTES, MCP-1 and MIP-1α, and NO. Third, lack of IL-12 synthesis by DC has a direct impact on IFN-γ production by lymph node cells as observed in MyD88 KO mice. Finally, we observed that TLR9 is an important molecule during *Brucella*
infection; however, the lack of MyD88 induces a more profound effect in host susceptibility. In conclusion, impaired DC maturation and reduced IL-12 production are major factors affecting host susceptibility to B. abortus infection in MyD88 KO mice.

Materials and Methods

Mice

MyD88, TLR2, TLR4, and TLR9 KO mice were provided by S. Akira (Osaka University, Osaka, Japan). The wild-type strain C57BL/6 mice were provided by Federal University of Minas Gerais (UFMG; Belo Horizonte, Brazil). Genetically deficient and control mice were maintained at UFMG and used at 6–8 wk of age. All animal experiments were preapproved by the Institutional Animal Care and Use Committee of the UFMG.

Bacteria

B. abortus virulent strain 2308 was obtained from our own laboratory collection (15). They were grown in Brucella broth medium (BD Biosciences) for 3 days at 37°C.

Infection and Brucella counts in spleens

Mice were infected i.p. with 10^6 CFU of B. abortus strain 2308. To count residual Brucella CFU in the spleens of mice, five animals from each group were examined at each sampling period. Splenocytes from individual animals were homogenized in PBS, 10-fold serially diluted, and plated on Brucella broth agar (Diñco). Plates were incubated at 37°C and the number of CFU was counted after 3 days as previously described (15).

Cytokine production in macrophage and lymph node cells

For macrophages, MyD88 KO, TLR9 KO, and C57BL/6 mice were inoculated i.p. with 2 ml of 3% thiglycolate (Diñco) and, 4 days later, the elicited peritoneal exudate cells were harvested in cold serum-free RPMI 1640. The medium used in macrophages cultures consisted of RPMI 1640 (Invitrogen Life Technologies) supplemented with 2 mM l-glutamine, 25 mM HEPES, 5% heat-inactivated FBS (Sigma-Aldrich), penicillin G sodium (100 U/ml), and streptomycin sulfate (100 µg/ml; supplemented RPMI 1640). The macrophages were washed and resuspended in supplemented RPMI 1640 at 10^5/ml and dispensed into the wells of a 96-well plate. Cells were allowed to adhere at 37°C and 5% CO_2 for 3 h, washed once with serum-free RPMI 1640, and 200 µl of supplemented RPMI 1640 was added to each well, in the presence or absence of 20 U of IFN-γ/ml. The cultures were stimulated by addition of 10^5 heat-killed bacteria (heat inactivation was performed at 80°C for 2 h)/ml, 1 µg/ml Escherichia coli LPS (Sigma-Aldrich), zymosan (50 µg/ml), or CpG (1 µg/ml) in a total volume of 200 µl of medium/well. For lymph node cells, freshly removed lymph nodes (inguinal, mesenteric, axillary, and brachial) of mice infected with B. abortus S2308 were placed in petri dishes containing 5 ml of PBS and passed through steel mesh to obtain single-cell suspensions. The lymph node cell suspension was then transferred to a sterile 15-ml tube. Cells were then washed twice with sterile PBS containing penicillin G sodium (100 U/ml), streptomycin sulfate (100 µg/ml). Afterward, lymph node cells were cultured in supplemented RPMI 1640 and plated at 10^5 cells/well in 96-well tissue-culture plates. The murine lymph node cells were stimulated with 1 µg/ml E. coli LPS (Sigma-Aldrich), zymosan (50 µg/ml), or 10^5 heat-inactivated B. abortus/well. Unstimulated cells were used as a negative control, and cells stimulated with Con A (5 µg/ml; Sigma-Aldrich) were a T-cell-activating control. These preparations were incubated at 37°C and 5% CO_2. Levels of TNF-α, IFN-γ, IL-12 (p40), IL-4, RANTES, MIP-1α, and MCP-1 in the supernatants were measured by a commercially available ELISA Duoset kit (R&D Systems).

NO measurement

To assay the amount of NO produced, macrophage culture supernatants from MyD88 KO, TLR9 KO, or C57BL/6 mice were assayed for accumulation of the stable end product of NO, NO_2^- which was determined by the Griess reaction. Briefly, culture supernatants (50 µl) were mixed with 30 µl of Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride, and 2.5% phosphoric acid) in triplicate in 96-well plates at room temperature for 5 min. The OD at 550 nm was then measured. NO_2^- was quantified by comparison with Na (NO_2^-; Sigma-Aldrich) as a standard.

Generation of BM-derived and splenic DCs

DCs were generated from BM mononuclear cells from wild-type C57BL/6, TLR9 KO, or MyD88 KO mice, in medium containing rGM-CSF as previously described (16). Briefly, femurs and tibiae were collected from mice with 12 wk old. After removing bone adjacent muscles, marrow cells were extracted by flushing RPMI 1640 medium through the bone interior. Unwanted fragments were removed by filtering the marrow suspension through 70-µm cell strainers (Falcon; BD Biosciences). Bone marrow cells were then resuspended on DC culture medium (RPMI 1640 medium, 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin/streptomycin, 50 mM 2-ME, 200 U GM-CSF), and plated on 100 mm petri dishes (1 × 10^6 cells/ml DC cell concentration). On days 2 and 4, the cells were refed. On day 5, cells were removed from petri dishes, plated on 6-well plates (3 × 10^5 cells/well) in 3 ml of fresh DC culture medium. After overnight culture, on day 6, cells demonstrated differentiated morphology and expressed DC markers (CD11c^+1, MHC class II (MHCII)^+ and CD40^+; data not shown), confirming to be immature DCs. For splenic DC, C57BL/6, TLR9 KO, TLR2 KO, or MyD88 KO mice were inoculated with 10^5 HKBA and the splenocytes were removed 24 h later. Then, spleen cell suspensions were treated with appropriate Abs for FACS analysis as described below.

FACS analysis and anti-TLR-blocking assay

On day 6, BMDCs were stimulated for 24 h with E. coli LPS (1 µg/ml), HKBA (10^5 bacteria/cell), zymosan (50 µg/ml), or CpG (1 µg/ml). Following in vitro stimulation for BMDCs or in vivo HKBA inoculation for splenic DC (24 h), cells were stained with fluorescein (FITC)-coupled HIL3 (anti-CD11c); PE-coupled APF-121 (anti-MHCII), and biotin-conjugated GL1 (anti-CD40) and 3/23 (anti-CD86) from BD Biosciences. Samples were then fixed and analyzed on a FACScan flow cytometer (BD Biosciences). Acquired data were analyzed by using FlowJo software (Tree Star). Additionally, 2 × 10^5 differentiated BMDCs or macrophages were plated on 96-well plates and incubated with blocking Abs anti-TLR2 at 5 µg/ml (Cell Sciences), and/or anti-TLR4 at 20 µg/ml (Imgenex) for 1 h before followed by Ag stimulation as indicated above. After BMDc and macrophage culturing, the cell-free supernatant was collected and stored until assay for IL-12 production.

MyD88 KO mice treatment with recombinant cytokines

MyD88 KO mice were i.p. injected daily with mouse rTNF-α (0.5 µg; PeproTech) in 0.2 ml of PBS, starting at 3 h before infection. Control mice were injected with PBS only. Additionally, MyD88 KO mice were treated 36 h prior infection with 1 × 10^9 PFU of a recombinant nonreplicating adenovirus expressing murine IL-12 (Ad5mIL-12) diluted in RPMI 1640 plus 1% of normal mouse serum in a total volume of 100 µl. This Ad5-based recombinant virus was gifted by Dr. F. L. Graham (McMaster University, Ontario, Canada) and it contains the p35 subunit of cDNA of murine IL-12 in early region 1 of adenovirus type 5 and the cDNA for p40 in early region 3 as previously described by Bramson et al. (17). This IL-12-expressing recombinant virus has also protected mice against lethal Klebsiella pneumoniae (18). The wild-type group were infected i.p. with 10^6 CFU of B. abortus strain S2308 on day 0 and residual Brucella CFU in the spleens were determined 1 wk after treatment.

Percentage of Ag-specific T cells producing IFN-γ

For intracytoplasmatic cytokine staining, splenocytes from MyD88 KO or C57BL/6 mice 2 wk infected with Brucella (1 × 10^6 CFU/mice) were adjusted to 5 × 10^7 cells/well. Splenocytes were maintained in culture at 37°C for 24 h. For stimulation, HKBA S2308 (10^3 bacteria/ml; Sigma-Aldrich) was added to impair cytokine secretion. After 3 h of incubation, these cells were stained for markers and intracellular cytokines. Briefly, cultured cells were incubated for 15 min with Fc-block (FcRII/III; BD Biosciences) in FACS buffer (0.15 M PBS, 2% BSA, 1% NaN_3) and stained for surface markers using fluorochrome-conjugated Abs: anti-MHCII (BioLegend), anti-CD11c (BD Biosciences), anti-CD4 (BioLegend), and anti-CD8 (BioLegend). After 15 h of culture, cells were washed twice with sterile PBS containing 0.05% saponin and fixed in 4% paraformaldehyde solution. These cells were permeabilized and further stained with 0.1% saponin in PBS, 2% BSA, 1% NaN_3, and stained for intracellular markers using fluorochrome-conjugated Abs: anti-IFN-γ, anti-IL-4, anti-IL-2 (from BD Biosciences, 0.5 µg/ml each). After fixing and permeabilization, these cells were incubated with FITC-conjugated antibodies against CD4 or CD8 (BD Biosciences, 0.5 µg/ml each) or isotype-matched control (BD Biosciences, 0.5 µg/ml each). Stained cells were then washed, resuspended in 1% paraformaldehyde, and analyzed on a FACScan flow cytometer.
MyD88 is a pivotal molecule in innate immunity against Brucella, functioning as an important molecule in the control of infection. MyD88 is required for efficient control of B. abortus in vivo. To check whether the reduction in IL-12 or TNF-α production is MyD88 dependent, lymph node cells from Brucella-primed C57BL/6 and MyD88 KO were cultured in the presence of medium, HKBA (10^9 organisms/well), E. coli LPS (1 μg/ml), or zymosan (50 μg/ml) and IFN-γ (A) or TNF-α (B) was measured by ELISA. Significant differences from C57BL/6 cells in relation to MyD88 KO are denoted by an asterisk (*) and from MyD88 KO cells stimulated with zymosan in relation to nonstimulated cells are denoted by two asterisks (**) for p < 0.05.

**Results**

**MyD88 is pivotal to control murine brucellosis**

To determine the contribution of MyD88 in bacterial clearance, numbers of Brucella were monitored in the spleens of MyD88 KO and C57BL/6 mice at 1, 2, 3, and 6 wk following B. abortus infection. The animals were sacrificed weekly and the numbers of CFU were determined. Murine brucellosis was markedly exacerbated in MyD88 KO mice at all intervals studied (Fig. 1). The difference in bacterial numbers in MyD88 KO and C57BL/6 was accentuated at 3 and 6 wk postinfection, 1.9 logs and 1.74 logs, respectively. These in vivo findings reinforce the role of MyD88 in innate immunity against Brucella, functioning as an important molecule in the control of infection.

**Lack of proinflammatory cytokine production by macrophages and lymph node cells parallels enhanced susceptibility of MyD88 KO mice**

IL-12 or TNF-α in contrast, wild-type macrophages produced ~800 or 2000 pg/ml IL-12 or TNF-α, respectively, when stimulated with HKBA (Fig. 3, A and B). Additionally, the level of IL-12 produced by wild-type mouse macrophages was influenced by the activation with IFN-γ. Furthermore, reduced levels of NO were observed in MyD88 KO macrophages stimulated with HKBA when compared with C57BL/6 macrophages (Fig. 3C). Regarding inflammatory chemokines, low levels of RANTES, MCP-1, and MIP-1α were detected in MyD88 KO macrophages when stimulated with HKBA compared with C57BL/6 (data not shown).

**Impairment of MyD88 KO Ag-specific T cells to produce IFN-γ**

To check whether the reduction in Brucella CFU observed in MyD88 KO mice during the course of infection (Fig. 2) is dependent on IFN-γ produced by Ag-specific T cells, we determined the percentage of CD4^+ or CD8^+ T lymphocytes producing IFN-γ following activation with recombinant B. abortus L7/L12, Omp16, and Omp19 proteins by FACS analysis. These Brucella proteins are described as major immunogenic molecules for this bacterium (26). As demonstrated in Table I, Brucella-primed CD4^+ or CD8^+ T cells from MyD88 KO mice were not able to produce IFN-γ when stimulated with recombinant proteins. In contrast, CD4^+ T cells from C57BL/6 mice produced significant levels of this cytokine to rL/L12, rOmp16, and rOmp19 proteins. This finding reveals that Th1 cell activation to specific Brucella proteins is impaired in MyD88 KO.
MyD88 is required for DC maturation following activation with HKBA

To characterize the role of MyD88 in DC maturation, we stimulated BM-derived DC with *E. coli* LPS or HKBA and analyzed cell surface expression of MHCII, CD40, CD86, on CD11c<sup>+</sup> cells. As demonstrated in Fig. 4, DCs from C57BL/6 mice stimulated with *E. coli* LPS or HKBA showed enhanced expression of MHCII (Fig. 4A), CD86 (Fig. 4B), and CD40 (Fig. 4C), compared with nonstimulated cells. A similar activation profile was observed in MyD88 DCs when stimulated with *E. coli* LPS. However, no signs of DC maturation were observed when MyD88 DCs were stimulated with HKBA demonstrating that HKBA-induced DC maturation is dependent of MyD88.

Because DC populations are heterologous (27, 28) and distinct subsets have been discriminated on the basis of experimental expression of various cell surface markers, we also evaluated the role of MyD88 on maturation of splenic DC in vivo (Fig. 5).

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**Table 1. Percentage of CD4<sup>+</sup> or CD8<sup>+</sup> T cells producing IFN-γ in response to Brucella recombinant proteins or HKBA in MyD88 KO and C57BL/6 mice**

<table>
<thead>
<tr>
<th></th>
<th>Medium</th>
<th>L7L12 OMP16/19</th>
<th>HKBa</th>
<th>Con A</th>
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</thead>
<tbody>
<tr>
<td>CD4/IFN-γ</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/6</td>
<td>1.46 ± 0.35</td>
<td>2.26 ± 0.5&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>3.26 ± 0.5&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>3.36 ± 0.7&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>MyD88&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>0.86 ± 0.05</td>
<td>0.8 ± 0.1</td>
<td>1.7 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.0 ± 0.17&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CD8/IFN-γ</td>
<td></td>
<td></td>
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<tr>
<td>C57BL/6</td>
<td>1.06 ± 0.15</td>
<td>1.2 ± 0.26</td>
<td>2.3 ± 0.45&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>3.76 ± 0.49&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>MyD88&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>0.53 ± 0.14</td>
<td>0.7 ± 0.3</td>
<td>0.5 ± 0.26</td>
<td>1.53 ± 0.12&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
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<sup>a</sup> Statistically significant compared to nonstimulated cells from the same animal.

<sup>b</sup> Statistically significant compared to MyD88 KO mice.
This result confirms our finding with BMDC that MyD88 deficiency impaired splenic DC maturation following in vivo HKBA stimulation. Additionally, we observed that splenic DC maturation is independent of TLR2 and TLR9 signaling.

Production of TNF-α and IL-12p40 by DCs after HKBA stimulation is MyD88 dependent

To assess the role of MyD88 on DC activation by HKBA, immature DCs were generated in vitro by culturing BM cells with GM-CSF. Additionally, we measured proinflammatory cytokine production in MyD88 KO and wild-type DC following HKBA stimulation. As shown in Fig. 6, IL-12 (Fig. 6A) and TNF-α (Fig. 6B) production by DC were totally abrogated in MyD88 KO mice when compared with C57BL/6 animals. These results confirmed that MyD88 is essential for HKBA-induced proinflammatory cytokine production by BMDC.

Production of IL-12p40 by DCs but not macrophages is TLR2 dependent

To determine whether TLR2 or TLR4 is involved in IL-12 production by DC or macrophages, cells from TLR2 KO, TLR4 KO, or C57BL/6 mice were used in this assay. For DC, cells of TLR2 KO and TLR4 KO mice were stimulated with medium, HKBA (10³ organisms/cell), E. coli LPS (1 μg/ml), or zymosan (50 μg/ml). Significant differences in relation to nonstimulated cells are denoted by * and to C57BL/6, TLR4 KO, HKBA, or HKBA + aTLR4 are denoted by #, for p < 0.05.
or TLR4 KO were incubated with E. coli LPS or HKBA. As observed in Fig. 7A, TLR2 is required for production of IL-12 by DCs stimulated with HKBA. Regarding macrophages, IL-12 production in this cell type is independent of TLR2 and TLR4 (Fig. 7B). These results were confirmed by blocking TLR2 or TLR4 receptor on DC or macrophages by mAbs (Fig. 7, C and D).

MyD88 KO treatment with rIL-12 enhances resistance to Brucella

To determine whether the greater susceptibility of MyD88 KO mice to brucellosis is due to a lesser ability to produce IL-12 or TNF-α in response to Brucella, these mice were treated with rTNF-α or recombinant adenovirus-expressing murine IL-12 (Ad5mIL-12). This strategy using a nonreplicating adenovirus-expressing murine IL-12 has shown to be more effective than the direct administration of rIL-12 protein (18). After 1 wk of infection, there was a statistically significant reduction in splenic Brucella CFU of MyD88 KO mice treated with rIL-12 but not with rTNF-α (Fig. 8). This result demonstrated that lack of IL-12 production in MyD88 KO is partially responsible for enhanced susceptibility to brucellosis observed in these animals.

TLR9 is required to control Brucella infection in vivo

To determine the role of TLR9 in Brucella infection in vivo, TLR9 KO mice were infected with virulent strain 2308. As demonstrated in Fig. 9, TLR9 KO mice showed enhanced susceptibility to B. abortus infection (log 6.16) compared with C57BL/6 mice (log 5.02) after 2 wk postinfection. However, TLR9 KO mice are more resistant than MyD88 KO animals. This result reflects the involvement of other TLRs that signal through MyD88.

Production of IL-12p40 by macrophage and DCs but not TNF-α is partially dependent on TLR9

To determine whether TLR9 played a role in IL-12 or TNF-α production, we stimulated macrophages or DC with HKBA, E. coli LPS or CpG. IL-12 but not TNF-α synthesis was reduced on either macrophages or DC activated with HKBA (Figs. 10 and 11). In contrast, induction of IL-12 by E. coli LPS, a TLR4 agonist, was not reduced in TLR9 KO mice. Additionally, NO production was intact on TLR9 KO macrophages stimulated with HKBA compared with C57BL/6 (Fig. 10C). These findings indicate that IL-12 production induced by HKBA on macrophages and DC is partially dependent on TLR9.
the predominant role of MyD88 in host resistance to *Brucella* infection in vivo and also in other intracellular microbial pathogens, IL-12-derived IFN-γ is critical on Th1 cell activation. In contrast to our findings, production of chemokines such as RANTES during *Mycobacterium tuberculosis* and *Pseudomonas aeruginosa* infection is MyD88 independent (32, 33). The lack of chemokine production may be another important deficiency in MyD88 KO macrophages when activated by HKBA that could enhance host susceptibility to murine brucellosis.

DCs are professional APCs and the major cell type involved in IL-12 production. Because IL-12 is critical for Th1 cell differentiation and IFN-γ production by CD4⁺ T cells, we investigated the role of MyD88 in DC maturation and activation by *B. abortus*. First, we confirmed that HKBA induces DC maturation in C57BL/6 mice by increased expression of CD40, CD86, and MHCII molecules on CD11c⁺ BM or splenic DC. In contrast, DC maturation was totally abrogated on MyD88 KO cells when HKBA was used as stimulus. This result was confirmed when in vivo splenic DC maturation was impaired in MyD88KO mice that received HKBA but not in TLR2 KO or TLR9 KO animals. Conversely, *E. coli* LPS could induce functional maturation of MyD88-deficient DC as previously reported (34). The inhibition of *Brucella*-induced DC maturation in MyD88-deficient mice argues in favor of a critical role for MyD88 in the detection of *B. abortus* by DC. Therefore, these results suggest that recognition of *Brucella* products by TLRs might be a prerequisite for MyD88-dependent DC maturation. Additionally, we measured IL-12 and TNF-α production in the supernatant of HKBA-stimulated BMDC, *E. coli* LPS, or zymosan-stimulated DCs. As shown in Fig. 6, production of these proinflammatory cytokines was totally abrogated in MyD88 KO DC either stimulated with HKBA or *E. coli* LPS but these cells were able to produce IL-12 and TNF-α when stimulated with zymosan.

To evaluate whether the lack of IL-12 or TNF-α in MyD88 KO could be responsible for reduced resistance to brucellosis, we treated these mice with rTNF-α or rIL-12. Administration of rIL-12 enhanced resistance of MyD88 KO mice to *Brucella* while rTNF-α therapy did not influence the outcome of infection. This result demonstrated that lack of IL-12 production in MyD88 KO is partially responsible for enhanced susceptibility to brucellosis observed in these animals.

To determine the TLR requirement for IL-12 production by DC stimulated with *Brucella*, we first investigated the role of TLR2 and TLR4. Previous studies reported that IL-12 secretion by splenic DC exposed to HKBA is TLR2/TLR4 independent but TLR9 dependent (12, 13). However, in our experiments, we observed that IL-12 production by BMDC was MyD88, TLR2, and TLR9 dependent. Similar results were obtained using anti-TLR treatment on C57BL/6 DC. This contrasting result may be due to different DC subsets used (35–37). The present study used BMDC, while splenic DC were used by others. Furthermore, other investigators have demonstrated the involvement of TLR2 in TNF-α and IL-6 production by a human monocytic cell line stimulated by HKBA (38). However, when macrophages from MyD88 KO or TLR2 KO were studied after HKBA stimulation, IL-12 production was dependent of MyD88 and independent of TLR2 and TLR4. Therefore, we concluded that TLR signaling to induce IL-12 production by HKBA is regulated differently in BMDC and inflammatory macrophages. A similar finding was observed in BMDC and macrophages stimulated with *M. tuberculosis* (39). Additionally, we have observed that HKBA secretion of IL-12 by TLR9 KO macrophages or DC was only partially reduced, suggesting a
role for other receptors in IL-12 signaling. Also, TNF-α production by macrophages or DC and NO by macrophages from TLR9 KO was not altered when compared with wild-type mice.

Recently, Huang et al. (13) reported the involvement of TLR9 on Brucella-driven Th1 responses. They suggested that DC in T cell areas are stimulated by DNA from HKBA via TLR9 to secrete IL-12 that activates nearby T cells to differentiate as Th1. Our investigation of the role of TLR9 in host control of B. abortus in vivo demonstrated that TLR9 deficiency enhances susceptibility to B. abortus infection. However, in comparison to TLR9, MyD88 deficiency has a more predominant role in host resistance to Brucella which suggests the involvement of other receptors that signals through MyD88 during the course of infection. In summary, the scenario emerging from our study is that susceptibility of MyD88 KO mice to B. abortus infection is due to impaired DC maturation and lack of IL-12 production. Because DC maturation is a critical link between innate and adaptive immunity, MyD88-dependent signaling appears to be required for the development of IFN-γ-producing T cells and efficient control of Brucella infection (40, 41). This was confirmed by lack of IFN-γ production in MyD88 KO lymph node cells of B. abortus-infected mice. Furthermore, we demonstrated by flow cytometry that CD4+ T cells from MyD88 KO mice have a deficient production of IFN-γ when stimulated with Brucella-specific recombinant Ags. Taken together, this study provides new insights into how innate and adaptive immunity are orchestrated to control B. abortus infection.

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Disclosures
The authors have no financial conflict of interest.

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