MyD88, but Not Toll-Like Receptors 4 and 2, Is Required for Efficient Clearance of Brucella abortus

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It is not clear how the host initially recognizes and responds to infection by gram-negative pathogenic Brucella spp. It was previously shown (D. S. Weiss, B. Raupach, K. Takeda, S. Akira, and A. Zychlinsky, J. Immunol. 172:4463–4469, 2004) that the early macrophage response against gram-negative bacteria is mediated by Toll-like receptor 4 (TLR4), which signals in response to lipopolysaccharide (LPS). Brucella, however, has a noncanonical LPS which does not have potent immunostimulatory activity. We evaluated the kinetics of TLR4 activation and the cytokine response in murine macrophages after Brucella infection. We found that during infection of macrophages, Brucella avoids activation of TLR4 at 6 h but activates TLR4, TLR2, and myeloid differentiation factor 88 (MyD88) at 24 h postinfection. Interestingly, even though its activation is delayed, MyD88 is important for host defense against Brucella infection in vivo, since MyD88−/− mice do not clear the bacteria as efficiently as wild-type, TLR4−/−, TLR2−/−, or TLR4/TLR2−/− mice.

Toll-like receptors (TLRs) are among the first receptors to detect a microbial infection. They recognize conserved microbial components and signal for inflammation (2, 33). Lipopolysaccharide (LPS) and bacterial lipoproteins (BLP) are two such microbial components and are recognized by TLR4 and TLR2, respectively (3, 5, 35). Brucellae are gram-negative bacteria that are found worldwide and cause an inflammatory disease characterized by fever, fatigue, weakness, and weight loss (21). The highest incidence of disease is in developing countries and among people who have close contact with livestock. A chronic generalized infection may follow the initial infection and results in part from the close contact with livestock. A chronic generalized infection is delayed, MyD88 is required for inflammation and efficient clearance of Brucella in vivo.

MATERIALS AND METHODS

Reagents. Highly purified Salmonella enterica serovar LPS was from List Biologicals (Campbell, CA). Brucella abortus LPS was prepared from phenol-water extracts of whole bacteria, and the crude material was extensively purified and characterized as described elsewhere (4, 12, 25). The purified Brucella LPS was free of contaminants and displayed the characteristic quantities of N-formyl-mercapto-O polysaccharide, 2-keto-3-deoxyoctulosonic acid, and the diaminoglucone backbone acylated with long-chain C28:0 to C30:0 fatty acids (28, 29). Murine TNF-α and murine interleukin-12 (IL-12) p40 enzyme-linked immunosorbent assays (ELISAs) were from R&D Systems (Minneapolis, MN), and the murine IL-10 ELISA was from BD Biosciences (San Diego, CA). The anti-mouse TLR4/MD-2 antibody was from eBioscience (San Diego, CA). ABTS [2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid)] was from Sigma Chemical Co. (St. Louis, Mo.).

Bacterial strains. Overnight cultures of Brucella abortus strain 19 (25) were prepared by thawing and adding a glycerol stock containing 1010 bacteria to 150 ml tryptic soy broth. Cultures were grown with shaking overnight at 37°C. Wild-type Salmonella enterica serovar Typhimurium SL1344 (17) was grown standing overnight at 37°C in high-salt Luria broth (LB; 0.3 M NaCl) supplemented with 200 μg/ml streptomycin.

Mice. Mice were bred under specific-pathogen-free conditions at New York University Medical Center, New York, or at the Bundesinstitut für Risikobewertung, Berlin, Germany. Mice were housed in filter-top cages and provided with sterile water and food ad libitum. TLR4−/− (19), TLR2−/− (40), MyD88−/− (22), IL-1B−/− (44), and IL-18−/− (39) mice have been described previously. We generated TLR4/TLR2−/− mice by crossing TLR4−/− and TLR2−/− mice, and IL-1B/IL-18−/− mice by crossing IL-1B−/− and IL-18−/− mice. All knockout mice were backcrossed at least seven times and were Nramp susceptible (Nramp+).
RESULTS

Brucella LPS has lower biological activity than Salmonella LPS. We incubated bone marrow-derived macrophages with LPS from Brucella or Salmonella to compare their activities. At 10 ng/ml, Salmonella LPS induced TNF-α release from wild-type macrophages (Fig. 1). In contrast, 50 μg/ml of Brucella LPS was required to induce a similar TNF-α response. Smaller amounts of Brucella LPS were insufficient to induce a comparable level of TNF-α secretion. These data indicate that Brucella LPS is 5,000-fold less active than Salmonella LPS as measured by TNF-α secretion. These results agree with previous observations (8, 14).

Slow induction of cytokines by Brucella-infected macrophages. To determine the timing of the macrophage response to Brucella infection, we infected macrophages with Brucella and measured cytokine production. Macrophages produced relatively low levels of TNF-α 24 h after Brucella infection but no detectable TNF-α at the earlier time points tested (Fig. 2A). This was in striking contrast to the strong induction of TNF-α seen at 2, 6, and 24 h after Salmonella infection (Fig. 2A). IL-12p40 and IL-10, two other cytokines induced upon TLR activation, were produced 24 and 48 h after Brucella infection, respectively (Fig. 2B and C). However, both cytokines were induced 6 h after Salmonella infection, and higher levels were produced during the experiment (Fig. 2B and C). These results show that the macrophage response to Brucella infection is delayed and less robust compared to that to Salmonella infection.

Down-regulation of TLR4 is delayed after Brucella infection. Activation of TLR4 down-regulates its own cell surface expression (31, 41). As an independent assay for TLR4 function, we tested the timing of TLR4 down-regulation after Brucella and Salmonella infection. Brucella infection induced TLR4 down-regulation at 24 h but not at 6 h (Fig. 3). Salmonella infection, however, down-regulated TLR4 by 6 h (Fig. 3). These results show that in addition to slow induction of cytokines, Brucella induces the down-regulation of TLR4 more slowly than Salmonella. We next investigated whether TLRs mediate the activation of macrophages by Brucella.
levels of TNF-α than wild-type macrophages (Fig. 4). TLR4/TLR2−/− and MyD88−/− cells did not produce significant amounts of TNF-α. These results demonstrate that the induction of TNF-α in response to Brucella is completely dependent on the combination of TLR4 and TLR2, as well as MyD88.

**MyD88 is required to control Brucella infection in vivo.** Since TLR4, TLR2, and MyD88 are involved in macrophage TNF-α production in response to Brucella infection in vitro, we tested their roles in host defense against Brucella in vivo. We infected mice intraperitoneally with 10⁶ CFU Brucella and quantified the number of bacteria in the spleen. Spleens from wild-type mice contained >10⁷ CFU/g on day 4 postinfection (Fig. 5A). On day 14 postinfection, the mice harbored 10⁶ CFU/g spleen, and on day 42 postinfection, they contained between 10⁴ and 10⁵ CFU/g spleen, showing that they were progressively clearing the infection (Fig. 5B and C). TLR4−/−, TLR2−/−, and TLR4/TLR2−/− mice were also able to effectively fight the infection and contained similar levels of Brucella as wild-type mice on each day tested (Fig. 5).

MyD88−/− mice harbored similar numbers of Brucella as wild-type mice on day 4 postinfection but, strikingly, did not begin to clear the infection by day 14, when their spleens contained 60 times more bacteria than wild-type spleens (Fig. 5A and B). On day 42 postinfection, MyD88−/− spleens contained fewer bacteria than on day 14 but still harbored 10 times more bacteria than wild-type spleens (Fig. 5C). Taken together, these results show that MyD88, but not TLR4 or TLR2, is required for efficient host defense against Brucella infection in vivo.

In addition to its role in the TLR pathway, MyD88 is required for IL-1β and IL-18 signaling (1). To test if these two cytokines contribute to the phenotype of the MyD88−/− mice, we infected IL-1β, IL-18, and IL-1β/IL-18 knockout mice with Brucella. On day 14, when the difference in bacterial burden between wild-type and MyD88−/− mice was most pronounced, IL-1β−/−, IL-18−/−, and IL-1β/IL-18−/− mice harbored similar levels of bacteria as wild-type mice (Fig. 6). These results show that IL-1β and IL-18 are not required for host defense against Brucella infection. The phenotype of the MyD88−/− mice is therefore likely due to defects in signaling from TLRs other than TLR4 and TLR2 or from another, unknown receptor.

**MyD88−/− mice have decreased splenic inflammation after Brucella infection.** To further understand the phenotype of the...
MyD88−/− mice, we examined sections of spleens from mice on day 14 postinfection. Infected wild-type spleens showed significant inflammation, with large numbers of infiltrating cells, and the normal splenic architecture was disrupted (Fig. 7A). In contrast, infected MyD88−/− spleens were less inflamed and contained fewer infiltrating cells, and the splenic architecture was preserved (Fig. 7B). These results suggest that the delayed clearance of Brucella by MyD88−/− mice can in part be explained by decreased inflammation.

MyD88 is not required to induce anti-Brucella LPS antibodies. Antibodies against Brucella LPS contribute to host defense in vivo (23, 30). We tested the levels of anti-Brucella LPS antibodies in the sera of infected mice on day 14 postinfection by ELISA. Wild-type mice had varying levels of anti-Brucella LPS antibodies, within a range of OD405s of 0.5 to 2.0 (Fig. 8). Similar results were seen for infected TLR4−/−, TLR2−/−, TLR4/TLR2−/−, and MyD88−/− mice. Uninfected mice did not contain anti-Brucella LPS antibodies. These results dem
onstrate that TLR4, TLR2, and MyD88 are not required for the induction of anti-Brucella LPS antibodies in vivo.

DISCUSSION

Slow activation of macrophages by Brucella. We show that Brucella activates macrophages 24 h postinfection but not 6 h postinfection, as measured by cytokine production and TLR4 down-regulation (Fig. 2 and 3). This is in contrast to Salmonella, which induces a strong macrophage response at both 6 and 24 h and induces TNF-α production as early as 2 h postinfection (Fig. 2 and 3). TLR4, which responds to LPS, is required for early macrophage activation during Salmonella infection (24, 37, 41). The lack of early macrophage activation by Brucella may be due to the 5,000-fold-lower activity of its LPS compared to that of Salmonella LPS (Fig. 1). Indeed, the structure of Brucella LPS strikingly departs from that of Salmonella, a fact consistent with its lower biological activity (29).

Interestingly, the combination of TLR4 and TLR2, as well as MyD88, is necessary and sufficient for induction of TNF-α production by Brucella 24 h postinfection (Fig. 4). It is not known which Brucella components activate TLR4 and TLR2. Brucella LPS may signal through TLR4. It is still an open question whether there is enough Brucella LPS during an infection to signal through TLR4, or if another Brucella component activates TLR4. If indeed Brucella LPS signals through TLR4, it is not clear why it signals at 24 h but not at 6 h, since TLR4 is expressed prior to and at 6 h. Brucella LPS may signal differently than classical enterobacterial LPS. Brucella LPS, in contrast to enterobacterial LPS, is not degraded by macrophages and instead accumulates in lysosomes up to 24 h postinfection and later recycles to the plasma membrane (11). Accumulation may be required for signaling, either from an intracellular location or from a plasma membrane location. These properties may explain the slow kinetics of Brucella LPS signaling in the context of an infection.

Brucella BLP may activate TLR2. Giambartolomei et al. described Brucella BLP as the active components in heat-killed Brucella, and Huang et al. showed that TLR2 is involved in the recognition of heat-killed Brucella (13, 20). Whichever Brucella components signal through TLR4 and TLR2, it is clear that both receptors are involved in the recognition of live Brucella.

MyD88, but not TLR4, TLR2, IL-1β, or IL-18, is required for efficient clearance of Brucella in vivo. Efficient clearance of Brucella infection requires MyD88, as evidenced by the fact that MyD88−/− mice harbor more bacteria than wild-type mice at days 14 and 42 postinfection (Fig. 5). These mice have reduced splenic inflammation, which may explain their delay in bacterial clearance (Fig. 7). Despite their roles in macrophage induction of TNF-α in vitro, TLR4 and TLR2 are not required for clearance of Brucella in vivo (Fig. 5). The lack of a role for TLR4 is in agreement with data from Huang et al., who showed that TLR4 is not required for TNF-α production in response to heat-killed Brucella in vivo (20). TLR4 is activated quickly by bacteria containing classical LPS, and its primary role in host defense may be in the initial recognition of microbes. The lack of early TLR4 activation by Brucella may explain why TLR4 is not required for host defense against the infection. We verified the lack of a role for TLR4 in another...
host genetic background by using C3H/HeJ mice, which have a naturally occurring, inactivating mutation in TLR4 (35). Consistent with our observations for TLR4 knockout mice, infected C3H/HeJ mice contained similar numbers of \textit{Brucella} as infected wild-type C3H/HeN mice (data not shown).

MyD88 is required for TLR signaling, as well as for IL-1β and IL-18 signaling. We find that IL-1β and IL-18 are not necessary for host defense against \textit{Brucella} infection (Fig. 6). Taken together, these results suggest that the phenotype of the MyD88 knockout mouse is likely due to a lack of signaling from TLRs other than TLR4 and TLR2, or from an as yet uncharacterized receptor.

Our data agree with the results of Campos et al., who showed that TLR2 does not play a role in host defense against \textit{Brucella} (6). However, using C3H/HeJ mice, Campos et al. observed that TLR4 might play a role in clearance of \textit{Brucella} (6). The discrepancy with our results may be due to the fact that Campos et al. used a different strain of \textit{Brucella} displaying lipid A fatty acid profiles that depart considerably from that of the \textit{Brucella} strain we used. Indeed, the characteristic long-chain fatty acids (C28:0 to C30:0), which are a hallmark of many alpha-2 proteobacterial LPSs, including that of \textit{Brucella} (28, 29), were not detected in their preparation (6).

Host defense against \textit{Brucella} infection depends in part on the antibody response (23, 30). We investigated whether the MyD88−/− phenotype was due in part to defects in antibody production. On day 14 postinfection, when spleens from MyD88 knockout mice contained more bacteria than spleens from wild-type mice, both groups of mice had similar levels of anti-\textit{Brucella} LPS antibodies (Fig. 8). This result suggests that the phenotype of the MyD88−/− mice is due to defects in innate immune function rather than to defects in antibody production. It will be interesting to test CD8 T-cell responses in \textit{Brucella}-infected MyD88 knockout mice, which also contribute to the host response against \textit{Brucella} (23).

Does \textit{Brucella} manipulate TLR function to find its niche? To live in host cells, \textit{Brucella} redirects the intracellular trafficking pathway, avoiding late endosomes and lysosomes, and localizing to membrane-bound compartments resembling the endoplasmic reticulum (ER) 6 to 8 h postinfection (7, 34). This is a mandatory step in the pathogenesis of \textit{Brucella}, since mutants that are defective in localization to the ER-like compartments are avirulent (7). The lack of early TLR4 signaling may buy time for the bacteria to redirect the intracellular trafficking pathway and localize to the ER-like compartments before the macrophage is effectively armed to fight the bacteria. By the time TLRs signal and the macrophage is activated, \textit{Brucella} is already in its intracellular niche. Therefore, the low activity of \textit{Brucella} LPS and subsequent evasion of early TLR4 signaling may be critical aspects of \textit{Brucella} pathogenesis.

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suggested the possible use of B. abortus or LPS from B. abortus as a carrier in vaccines. Infect. Immun. 60:1385–1389.


