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Brian T. Edelson and Emil R. Unanue

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MyD88-Dependent but Toll-Like Receptor 2-Independent Innate Immunity to *Listeria*: No Role for Either in Macrophage Listericidal Activity¹

Brian T. Edelson and Emil R. Unanue²

We have assessed the requirements for Toll-like receptor (TLR) signaling in vivo during early infection with *Listeria monocytogenes*. Mice deficient for TLR2, a receptor required for the recognition of Gram-positive peptidoglycan, showed equivalent *Listeria* resistance to wild-type mice. However, mice deficient for MyD88, an adaptor molecule used by all TLRs, showed profound susceptibility with 3–4 logs greater *Listeria* burden and severe spleen and liver pathology at day 3 postinfection. *Listeria*-infected MyD88-deficient mice also showed markedly diminished IFN- γ , TNF- α , and NO responses, despite evidence of macrophage activation and up-regulation of MHC class II molecules. We demonstrate that although minor MyD88-independent responses to live *Listeria* do occur, these are insufficient for normal host defense. Lastly, we performed experiments in vitro in which macrophages deficient in TLR2 or MyD88 were directly infected with *Listeria*. Although TLR signaling was required for macrophage NO and cytokine production in response to *Listeria*, handling and direct killing of *Listeria* by activated macrophages occurred by TLR2- and MyD88-independent mechanisms. *The Journal of Immunology*, 2002, 169: 3869–3875.

Toll-like receptors (TLRs)³ represent an evolutionarily conserved family of membrane proteins responsible for the recognition of diverse microbial products produced by bacteria, fungi, protozoa, and viruses (reviewed in Refs. 1–4). In mammalian cells, activation of TLRs results in stimulation of the innate immune system through well-described signaling pathways involving several adaptor molecules (notably MyD88), kinases, and transcription factors. These signals result in the up-regulation of cytokines, costimulatory molecules, and antimicrobial responses known to be required for both the early control of pathogens by the innate immune system and the induction of an appropriate adaptive immune response. Although several reports have elucidated the particular specificities of distinct TLRs, relatively few studies have assessed the requirements for mammalian TLR signaling during infection in vivo with live pathogens (5–10).

In this study, we describe the innate immune response of mice deficient in TLR signaling during infection with the Gram-positive, facultative intracellular bacterium *Listeria monocytogenes*. *Listeria* is a widely used model of intracellular bacterial infection, known to require virtually all aspects of the innate and adaptive immune responses for effective control (11). We used mice deficient in either TLR2, the receptor which as a heterodimer with TLR6 is responsible for recognition of Gram-positive peptidoglycan, or MyD88, the adaptor molecule required for all but a subset

of TLR-mediated signaling events (12–19). MyD88 also serves as an adaptor molecule in signaling via the IL-1 and IL-18 receptors, such that MyD88-deficient cells also lack responses to these cytokines (20–23).

Our results indicate a redundancy in the recognition of *Listeria* by TLRs, such that in the absence of TLR2 other molecules are sufficient for control of *Listeria* infection. However, MyD88 is absolutely required for early *Listeria* resistance and full activation of innate immune responses. We show that this requirement for MyD88 in *Listeria* resistance is in part due to its role in IL-1 and IL-18 signaling, but more importantly due to MyD88's role in TLR signaling. We demonstrate that while partial innate immune responses are stimulated by *Listeria* in a MyD88-independent fashion, these responses are insufficient for in vivo resistance. Lastly, we show that direct *Listeria* killing by activated macrophages occurs through a TLR2- and MyD88-independent mechanism, such that macrophage handling of *Listeria* is not altered by the absence of TLR signals.

Materials and Methods

Mice

C57BL/6 (Charles River Laboratories, Wilmington, MA), TLR2^{-/-}, MyD88^{-/-}, inducible NO synthase (iNOS)^{-/-} (on a C57BL/6 background; The Jackson Laboratory, Bar Harbor, ME), B6129SF1/J (The Jackson Laboratory), and Caspase 1^{-/-} mice were maintained and bred under SPF conditions in the Washington University mouse facility (St. Louis, MO) (12, 22, 24, 25). TLR2^{-/-} and MyD88^{-/-} mice, a kind gift of Dr. S. Akira (Osaka University, Osaka, Japan) via Dr. D. Golenbock (University of Massachusetts Medical School, Worcester, MA), were used after five generations of back-crossing to the C57BL/6 background, with C57BL/6 mice serving as controls. Caspase 1^{-/-} mice (originally generated at BASF Bioresearch, Worcester, MA), a kind gift of Dr. D. Chaplin (University of Alabama, Birmingham, AL), were on a mixed C57BL/6 \times 129Sv background, with B6129SF1/J mice serving as controls.

Bacteria

L. monocytogenes strains used in this study were the following: the wild-type (WT) strain EGD, the listeriolysin O (LLO) deletion mutant EJL1, and the WT parent strain of EJL1, 10403S (26). The EGD strain was used for all in vivo experiments, with an LD50 in C57BL/6 mice of $\sim 1 \times 10^6$ bacteria. *Listeria* was stored as glycerol stocks at -80°C and diluted into

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³ Abbreviations used in this paper: TLR, Toll-like receptor; LLO, listeriolysin O; BMDM, bone marrow-derived macrophage; HKLM, heat-killed *L. monocytogenes*; PEC, peritoneal exudate cell; iNOS, inducible NO synthase; WT, wild type; PMN, polymorphonuclear leukocyte.

pyrogen-free saline for injection into mice. For ex vivo use with bone marrow-derived macrophages (BMDMs), live *Listeria* was used as in Ref. 26. Heat-killed *L. monocytogenes* (HKLM; strain EGD) was prepared by incubation of mid-log bacteria at 70°C for 3 h followed by three washes with sterile PBS.

In vivo experiments

Listeria was used at a dose of 5×10^5 *Listeria*/mouse i.p., except where indicated. Mice were sacrificed at day 3 postinfection for determination of serum cytokine and nitrate/nitrite levels, assessment of peritoneal exudate cells (PEC), and quantitation of organ *Listeria* burden. PEC were collected by peritoneal lavage and stained for flow cytometry with FITC-conjugated anti-I-A^b (BD Pharmingen, San Diego, CA) and PE-conjugated anti-F4/80 (Caltag Laboratories, Burlingame, CA). For light microscopy, cells were cytospun onto slides and stained with the Hema 3 staining kit (Fisher Scientific, Pittsburgh, PA).

To determine organ *Listeria* burden, spleen and liver were homogenized in PBS plus 0.05% Triton X-100. Serial dilutions of homogenate were plated on brain heart infusion agar, and bacterial CFU were assessed after overnight growth at 37°C. Small portions of spleen and liver were also fixed in 10% formalin and stained with H&E.

Cytokine and nitrate/nitrite determinations

Serum IL-12 p40 levels were measured using the OptEIA ELISA set (BD Pharmingen). Serum IFN- γ and TNF- α levels were measured in ELISA using standard methods, with reagents provided by Dr. R. Schreiber (Washington University). Serum nitrate and nitrite levels were determined by converting nitrate to nitrite with aspergillus nitrate reductase (Sigma-Aldrich, St. Louis, MO), followed by nitrite measurement using the Griess reagent.

BMDM experiments

Bone marrow was collected from femurs of mice and cultured as described to generate BMDM (27). Briefly, cells were cultured for 6 days in complete DMEM containing 10% heat-inactivated FCS, 5% heat-inactivated horse serum, and 20% culture supernatant from L929 cells. After day 6, cells were cultured in the above media without L929 supernatant. To assess BMDM responses to HKLM, live *Listeria*, and LPS (*Escherichia coli* serotype O55:B5; Sigma-Aldrich), macrophages at day 8 of culture were stimulated in antibiotic-free media containing 300 U/ml murine IFN- γ in triplicate in 96-well plates at 5×10^4 cells/well. Two hours after addition of stimuli, penicillin and streptomycin were added to all wells. Supernatants were collected after 48 h and assessed for nitrite, TNF- α , and IL-12 p40 using the methods described above.

To assess intracellular *Listeria* growth in BMDM, cells were cultured for 48 h, beginning on day 8, in antibiotic-free media in the presence or absence of 300 U/ml IFN- γ . Cells were then plated at 2.5×10^5 cells/well on 12-mm glass coverslips in 24-well plates. Nonadherent cells were removed after a 2-h incubation at 37°C. Infection of cells, determination of CFU/coverslip at various time points postinfection, and assessment of intracellular *Listeria* localization at 4 h postinfection (scoring individual organisms as either "phagosomal" or "cytosolic"), were performed as described (26).

Results

MyD88-dependent but TLR2-independent *Listeria* resistance in vivo

WT, TLR2^{-/-}, and MyD88^{-/-} mice were infected with *Listeria* and sacrificed at day 3 postinfection (Fig. 1). TLR2^{-/-} mice showed normal immunity, with *Listeria* titers in both spleen and liver equivalent to WT mice. Histologic assessment of TLR2^{-/-} mice was identical with WT mice (data not shown). However, MyD88^{-/-} mice showed a profound deficiency in their innate immune response to *Listeria*, with an ~3-log greater spleen *Listeria* burden, and 4-log greater liver *Listeria* burden. Spleens from normal mice showed moderate lymphocyte depletion (as determined by the presence of pyknotic nuclei on light microscopy, consistent with Ref. 28) located centrally within white pulp regions, affecting <25% of follicles (Fig. 1C). MyD88^{-/-} spleens showed complete destruction of all white pulp follicles, with apoptosis extending throughout the follicles to the marginal zone (Fig. 1D). Livers of WT mice showed scattered, small foci of

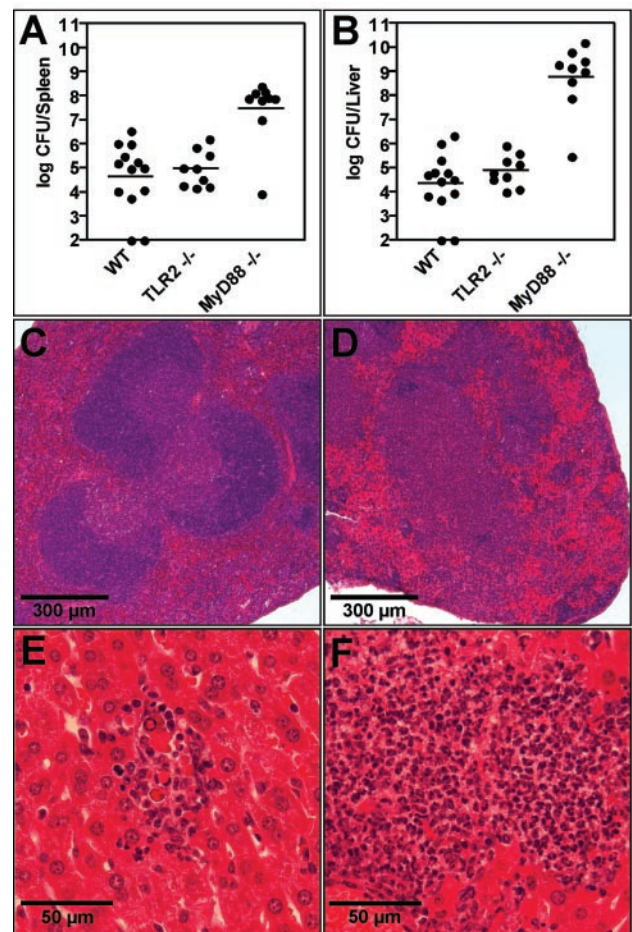


FIGURE 1. Infection of WT, TLR2^{-/-}, and MyD88^{-/-} mice with *Listeria*. Mice were infected with 5×10^5 *Listeria* i.p., and sacrificed at day 3 postinfection. A and B, CFU/organ. Circles represent individual mice, and bars represent geometric mean CFU/organ. Results shown are the combination of three identical experiments. Spleen: WT vs TLR2^{-/-}, $p = 0.7385$ (Mann-Whitney U test); WT vs MyD88^{-/-}, $p = 0.0014$. Liver: WT vs TLR2^{-/-}, $p = 0.3164$; WT vs MyD88^{-/-}, $p = 0.0002$. C–F, Representative H&E-stained sections from infected mice at day 3 postinfection. C, WT spleen, showing moderate lymphocyte depletion located centrally within white pulp follicles. D, MyD88^{-/-} spleen, showing complete destruction of follicle architecture. E, WT liver, showing an average-sized lesion consisting of macrophages surrounding a core of dead hepatocytes. F, MyD88^{-/-} liver, showing a large abscess consisting of extensive neutrophil infiltration.

infection with macrophages often surrounding a core of dead hepatocytes (Fig. 1E). MyD88^{-/-} livers showed much more numerous, large foci of infection consisting of extensive neutrophil infiltrates (Fig. 1F). Lung, kidney, and pancreas from three of four MyD88^{-/-} mice showed no signs of infection, with one mouse showing neutrophil infiltrates in the lung and patches of *Listeria* growth in the exocrine pancreas. MyD88^{-/-} mice were found to die at day 4 postinfection due to overwhelming bacteremia.

We compared *Listeria* infection in Caspase 1^{-/-} mice, which lack production of the mature forms of IL-1 α , IL-1 β , and IL-18 in response to inflammatory challenge (25). Caspase 1^{-/-} mice showed minimally increased spleen *Listeria* titers, and an ~2-log increase in liver titers (Fig. 2). We interpret these results to mean that while both IL-1 and IL-18 do play a role in the innate immune response to *Listeria* (29–31), MyD88 serves an even greater role in resistance by also providing TLR signals in response to *Listeria*.

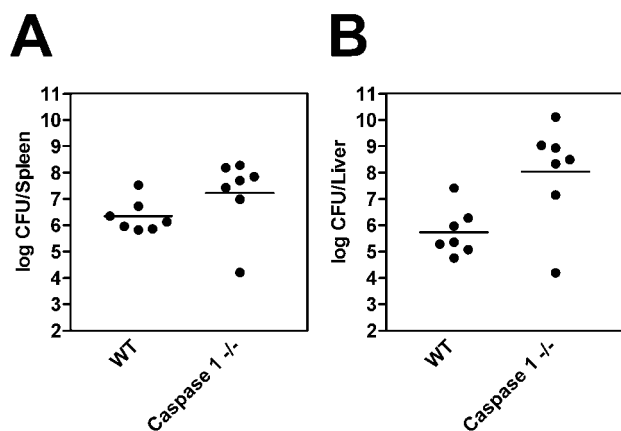


FIGURE 2. Infection of WT and Caspase 1^{-/-} mice with *Listeria*. Mice were infected with 5×10^5 *Listeria* i.p., and sacrificed at day 3 postinfection. *A* and *B*, CFU/organ. Circles represent individual mice, and bars represent geometric mean CFU/organ. Results shown are the combination of two identical experiments. Spleen, $p = 0.0530$. Liver, $p = 0.0379$.

Listeria-mediated TLR2- and MyD88-independent macrophage activation

PEC from uninfected and *Listeria*-infected WT, TLR2^{-/-}, and MyD88^{-/-} mice were examined by light microscopy and flow cytometry. Macrophages from all uninfected mice were of normal size and nonvacuolated, with high surface expression of F4/80 and low expression of the MHC class II molecule I-A^b (Fig. 3, *A* and *B*). Macrophages from infected WT and TLR2^{-/-} mice showed an activated phenotype, with an ~2-fold increase in size, membrane ruffling, and vacuolization (Fig. 3C, data not shown for TLR2^{-/-} mice). By flow cytometry, these cells were F4/80^{low} and I-A^b^{high}. Peritoneal macrophages from infected MyD88^{-/-} mice also showed an activated phenotype by both light microscopy and flow cytometry (an equivalent decrease in F4/80 and increase in I-A^b compared to WT cells), but were also hypervacuolated in comparison to WT cells (Fig. 3D). Several MyD88^{-/-} macrophages showed the presence of intracellular (often intravacuolar) *Listeria* organisms, with extracellular *Listeria* also present. *Listeria* was never evident in preparations of PEC from WT or TLR2^{-/-} mice.

Diminished inflammatory responses in Listeria-infected MyD88^{-/-} mice

We assessed several parameters of inflammation at day 3 postinfection in *Listeria*-infected WT and MyD88^{-/-} mice challenged with a dose of 5×10^5 organisms (Fig. 4, *left panels*). Because MyD88^{-/-} mice challenged with this dose had profoundly increased *Listeria* titers, we also performed a separate experiment in which WT mice were challenged with three graded doses of *Listeria* (5×10^5 , 5×10^6 , and 5×10^7), resulting in increasing *Listeria* titers at day 3 postinfection (Fig. 4, *right panels*). This provided us a way to measure whether the responses of MyD88^{-/-} mice were commensurate with their large *Listeria* burden. MyD88^{-/-} mice challenged with a dose of 5×10^5 *Listeria* had roughly equivalent bacterial titers to WT mice challenged with 5×10^7 organisms. It is noteworthy that three of four WT mice died before day 3 at this dose. MyD88^{-/-} mice appear to survive with higher bacterial burdens than WT mice before succumbing to infection, suggesting that death of WT mice is in part a consequence of the massive inflammatory response taking place.

The first parameter assessed was the percentage of neutrophils in the peritoneal exudates of infected mice (Fig. 4, *C* and *D*). MyD88^{-/-} showed a slightly diminished neutrophil response

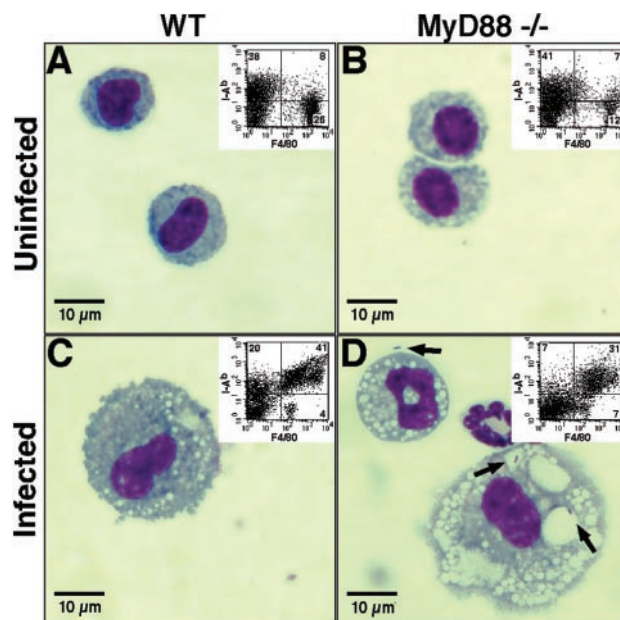


FIGURE 3. Peritoneal macrophages from both WT and MyD88^{-/-} mice show an activated phenotype upon *Listeria* infection. Mice were infected with 5×10^5 *Listeria* i.p. and sacrificed at day 3 postinfection. *A* and *B*, Peritoneal macrophages from uninfected WT (*A*) and MyD88^{-/-} (*B*) mice. Cells are of normal size and nonvacuolated. *Insets*, FACS plots of PEC show F4/80^{high}, I-A^b^{low} staining macrophages. *C* and *D*, Peritoneal macrophages from infected WT (*C*) and MyD88^{-/-} (*D*) mice. In both cases, cells show an activated phenotype with increased size and vacuolation. *D*, MyD88^{-/-} cells in fact show hypervacuolation and the presence of both intracellular and extracellular *Listeria* organisms (arrows). *D* also shows the presence of a neutrophil which was in greater abundance in PEC of MyD88^{-/-} mice infected with this dose of *Listeria* (see Fig. 4C). *Insets*, FACS plots of PEC show the majority of macrophages now staining F4/80^{low} and I-A^b^{high}.

(~25% polymorphonuclear leukocyte (PMN)) relative to their high *Listeria* titers vs WT mice (~30% PMN in mice challenged with the 5×10^6 dose, and ~40% PMN with the 5×10^7 dose).

The next parameters assessed were the serum levels of IL-12, IFN- γ , and TNF- α ; three cytokines known to be required for normal *in vivo* *Listeria* resistance (11). WT mice showed a small but detectable increase in serum IL-12 levels when challenged with 5×10^5 *Listeria* (Fig. 4, *E* and *F*). However, with increasing bacterial titers IL-12 levels decreased below basal levels (Fig. 4F). Infected MyD88^{-/-} mice also showed lower than basal levels of IL-12 (Fig. 4E). In light of the inverse relationship between IL-12 levels and bacterial titers in WT mice, we are unable to definitively say whether IL-12 production was abnormal in *Listeria*-infected MyD88^{-/-} mice.

Serum levels of both IFN- γ and TNF- α were increased above basal levels upon *Listeria* infection of MyD88^{-/-} mice (Fig. 4, *G* and *H*), indicating the existence of *Listeria*-induced MyD88-independent pathways of cytokine production *in vivo*. However, levels of both cytokines in MyD88^{-/-} mice were below the levels found in WT mice with equivalent *Listeria* titers (~100-fold decrease in IFN- γ and 10-fold decrease in TNF- α ; Fig. 4, *H* and *J*). IL-10 levels in serum from all mice shown in Fig. 4 were found to be virtually undetectable (data not shown), indicating no role for this anti-inflammatory cytokine in the susceptibility of MyD88^{-/-} mice.

Lastly, we assessed the serum levels of nitrate and nitrite as a marker of *in vivo* iNOS-mediated NO production, an inflammatory response known to be required for *in vivo* *Listeria* resistance (32–34). Infected MyD88^{-/-} mice showed a minimally increased level

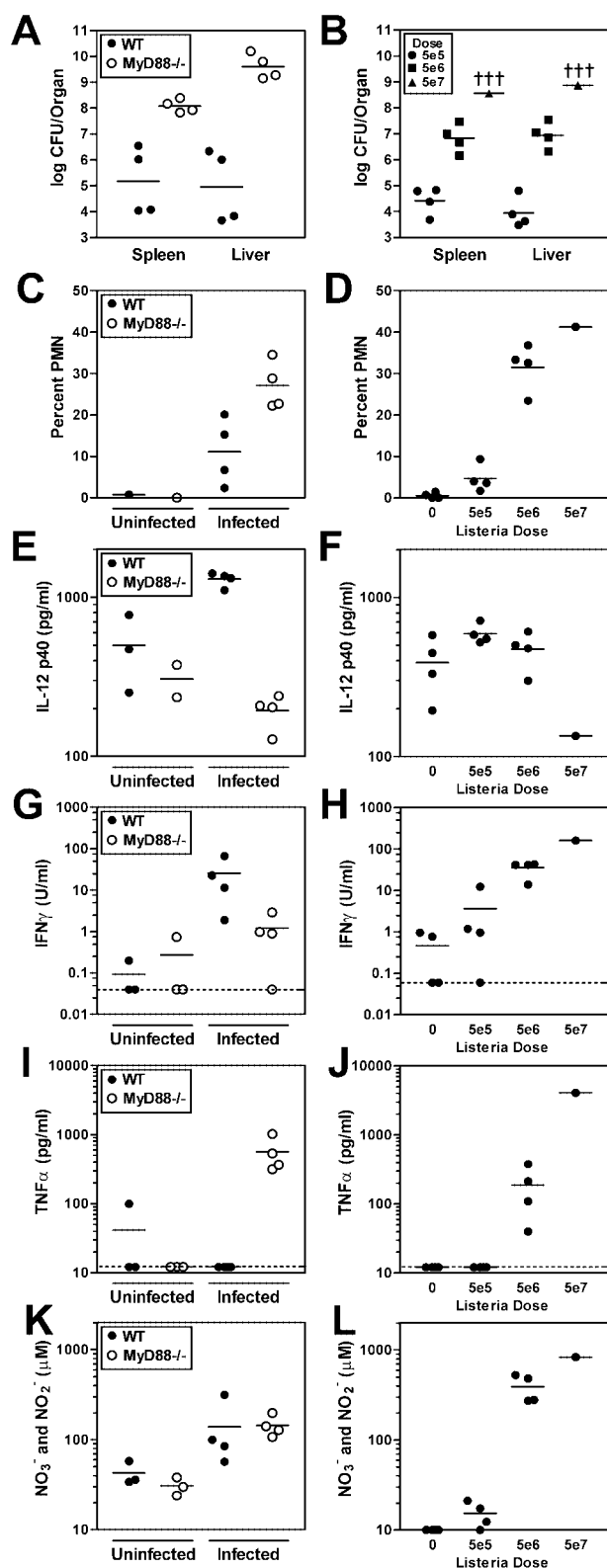


FIGURE 4. Assessment of inflammatory responses in WT and MyD88^{-/-} mice. *Left panels*, comparison of WT and MyD88^{-/-} mice infected with 5×10^5 *Listeria* i.p., and sacrificed at day 3 postinfection. *Right panels*, comparison of WT mice only, infected with 5×10^5 , 5×10^6 , or 5×10^7 *Listeria* i.p., and sacrificed at day 3 postinfection. In all cases, circles represent individual mice. *A and B*, CFU/organ. Note that in *B*, three of four WT mice infected with 5×10^7 *Listeria* died before day 3 postinfection. *C and D*, Percentage of PEC identified as neutrophils (PMN) by differential counting. *E and F*, Serum IL-12 levels. *G and H*, Serum IFN- γ levels. *I and J*, Serum TNF- α levels. *K and L*, Serum nitrate/nitrite levels.

of nitrate/nitrite in the serum (Fig. 4K). This small response was ~ 10 -fold decreased relative to WT mice harboring a similar *Listeria* burden (Fig. 4L), and also likely contributed to the susceptibility of MyD88^{-/-} mice.

Macrophage responses to *Listeria* in vitro: TLR2- and MyD88-dependent and -independent responses

To further characterize the inflammatory responses of macrophages to *Listeria*, we performed a series of in vitro experiments using BMDM derived from WT, TLR2^{-/-}, MyD88^{-/-}, and iNOS^{-/-} mice. NO, TNF- α , and IL-12 production were determined after stimulation with HKLM, live *Listeria*, and LPS, all in the presence of simultaneous stimulation with IFN- γ (Fig. 5, A–E). TLR2 was found to be absolutely required for stimulation by HKLM, but played only a very minor role with live *Listeria*. MyD88^{-/-} cells showed no responses to HKLM, yet showed small but detectable NO and TNF- α responses to both live *Listeria* and LPS (with ~ 100 -fold less sensitivity, and with lower maximums). As expected, iNOS^{-/-} cells showed no NO production but normal TNF- α and IL-12 responses to all stimuli.

These results demonstrate the following: 1) HKLM contains only ligand(s) capable of stimulating responses through TLR2 (likely peptidoglycan) (12, 35); 2) live *Listeria* contains ligand(s) for both TLR2 and probably other TLRs; 3) responses to live *Listeria* through non-TLR2 ligands require MyD88 for maximal responses, but can also induce partial responses in a MyD88-independent fashion (similar to MyD88-independent responses to LPS).

Listeria killing by IFN- γ activated macrophages is TLR2-, MyD88-, and iNOS-independent

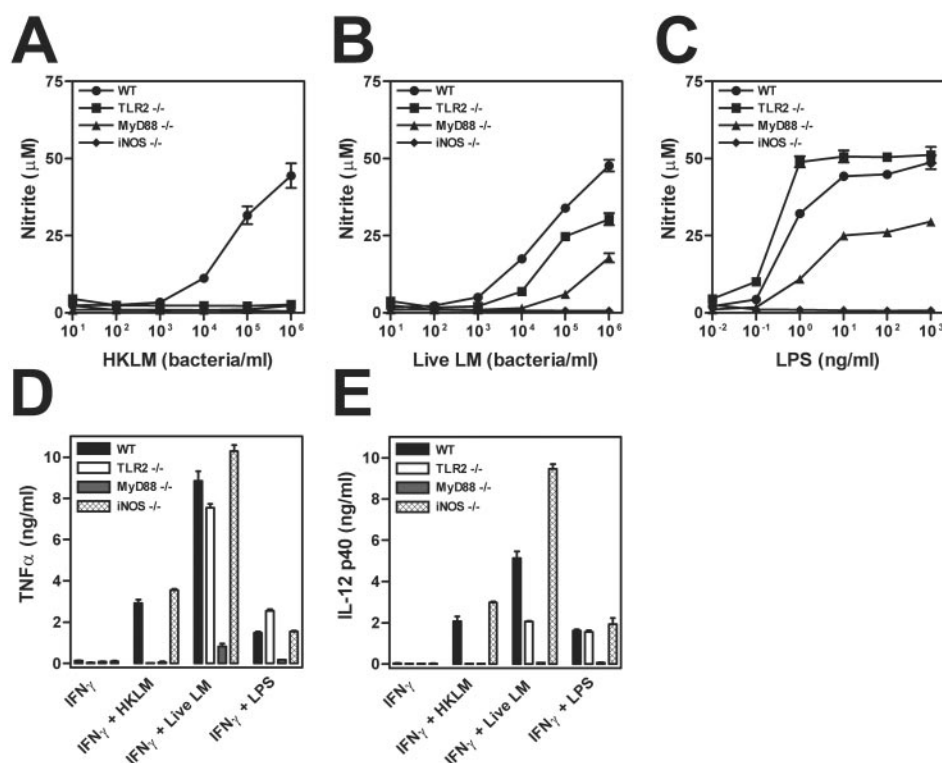
To determine whether signals through TLR2 or MyD88 contribute to *Listeria* killing by macrophages in vitro, experiments were performed in which the intracellular growth of *Listeria* was followed in BMDM in vitro (Fig. 6, A–C). In Fig. 6A, both resting and IFN- γ -activated BMDM from all strains of mice tested were shown to handle *Listeria* equivalently, with resting cells allowing significant intracellular bacterial growth and activated macrophages showing powerful bactericidal activity. This result demonstrates no role for TLR2, MyD88, or NO in *Listeria* killing induced by IFN- γ .

TLRs are known to localize to pathogen-containing phagosomes (36). Because intracellular *Listeria* growth depends upon the ability of *Listeria* to escape from phagosomes to the cytosol, and because IFN- γ acts to control *Listeria* growth by blocking this escape (37–40), we also measured the efficiency of *Listeria* escape to the cytosol in WT, TLR2^{-/-}, and MyD88^{-/-} cells (Fig. 6B). No differences were seen in BMDM in the absence of TLR2 or MyD88, indicating no role for the signals deriving from these molecules in direct *Listeria* handling by macrophages. Lastly, we compared the intracellular growth of WT (strain 10403S) vs LLO-deficient *Listeria* (strain EJL1, unable to escape to the cytosol due to deletion of LLO) in BMDM from WT and MyD88^{-/-} mice (Fig. 6C). Again, no role for MyD88 was seen in the killing of WT *Listeria* by activated macrophages, or in the killing of LLO-deficient *Listeria* by both resting and activated macrophages.

Discussion

In this report, we have investigated the requirements for TLR2 and MyD88 during in vivo infection with *Listeria*. TLR2-deficient mice showed normal *Listeria* resistance, while MyD88-deficient mice showed extreme susceptibility. In this study, we have shown by in vitro experiments that while TLR2 is required for responsiveness to HKLM, likely recognizing peptidoglycan still present

FIGURE 5. In vitro responses to *Listeria* by WT, TLR2^{-/-}, MyD88^{-/-}, and iNOS^{-/-} BMDM. A–C, BMDM from indicated mice were cultured for 48 h in the presence of IFN- γ with indicated doses of HKLM (A), live *Listeria* EGD (LM) (B), or LPS (C). Nitrite concentration was determined in supernatants (mean \pm SEM). D and E, BMDM from indicated mice were cultured for 48 h in the presence of IFN- γ alone, or in combination with HKLM (1×10^6 /ml), live *Listeria* EGD (LM, 1×10^6 /ml), or LPS (1 ng/ml). TNF- α (D) and IL-12 p40 (E) concentrations were determined in supernatants (mean \pm SEM).



after heat treatment, non-TLR2 ligand(s) exist on live *Listeria* which can efficiently stimulate TLR2-deficient cells. This redundancy of recognition of a complex live pathogen by the TLR system explains the in vivo resistance of TLR2-deficient mice to *Listeria* infection, and presumably serves the host to more effectively recognize pathogens. We hypothesize that TLRs 2, 4, 5, 6, and 9 may all be involved in the in vivo response to live *Listeria* through recognition of peptidoglycan (heterodimer of TLRs 2 and 6) (12, 13), lipoteichoic acid (TLR4; Ref. 12), *Listeria* flagellin (TLR5; Ref. 41), and bacterial DNA (TLR9; Ref. 42). The dissection of this redundancy will require the generation of mice deficient in several TLRs, or tools to pharmacologically inhibit specific TLRs.

It is noteworthy that TLR2-deficient mice were found to be susceptible to *Staphylococcus aureus* and *Borrelia burgdorferi*, suggesting that TLR2 does seem to be of primary importance during infection with these pathogens (5, 8).

MyD88-deficient mice were highly sensitive to *Listeria* infection, allowing uncontrolled bacterial growth in spleen and liver. We hypothesize that this overall susceptibility results from a combination of defects resulting from the lack of the MyD88 protein. IL-1 and IL-18, molecules whose signaling pathways require MyD88, have both been shown to be required for normal defense against *Listeria*, and indeed we found Caspase 1-deficient mice, which lack production of these cytokines, to have increased

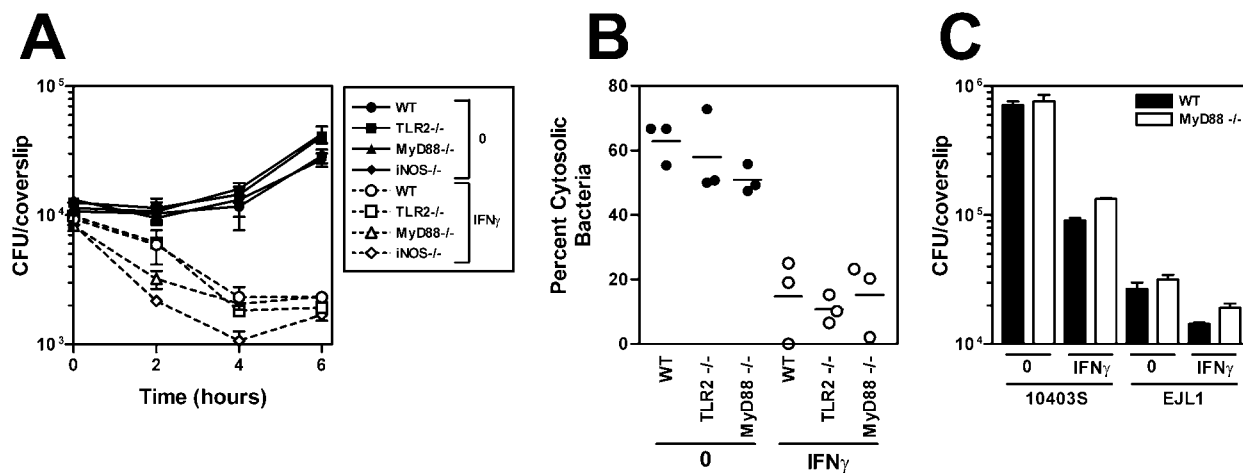


FIGURE 6. In vitro macrophage listericidal activity. A, BMDM (resting or activated with IFN- γ) from indicated mice were infected with live *Listeria* EGD, and CFU/cover slip (mean \pm SEM) were determined at times 0, 2, 4, and 6 h postinfection. B, BMDM (resting or activated with IFN- γ) from indicated mice were infected with live *Listeria* EGD, and the percentage of cytosolic bacteria was determined at 4 h postinfection. Each circle represents a randomly selected high power field of view in which the bacteria contained within the central 12 macrophages were evaluated (~ 30 – 50 bacteria per field). C, BMDM (resting or activated with IFN- γ) from WT or MyD88^{-/-} mice were infected with live *Listeria* 10403S (WT strain) or *Listeria* EJL1 (LLO-deficient strain). CFU/cover slip (mean \pm SEM) were determined at 6 h postinfection.

bacterial titers in the liver (20–23, 29–31). However, the susceptibility of Caspase 1-deficient mice was significantly less than that seen for MyD88-deficient mice. This implies a critical role for MyD88 beyond the IL-1 and IL-18 pathways, in other words acting as an adaptor for TLR-mediated signaling.

MyD88-deficient mice showed compromised inflammatory responses to *Listeria* infection, including diminished PMN responses and diminished IL-12, IFN- γ , TNF- α , and NO production. Each of these is independently known to be required for normal *Listeria* resistance; and therefore, the combination of these defects likely resulted in overwhelming infection (11). It is important to note that each of the above mentioned responses (with the exception of IL-12) is not absent in *Listeria*-infected MyD88-deficient mice, only reduced. This indicates that MyD88-independent responses are taking place in vivo, although at an insufficient level for normal host defense. Macrophage activation during in vivo infection, as determined by microscopic evaluation and assessment of surface levels of F4/80 and MHC class II molecules, was also seen to be an MyD88-independent response. Up-regulation of MHC class II molecules is known to require IFN- γ , suggesting that the low level of this cytokine induced in MyD88-deficient mice is sufficient to mediate this response (43).

In vitro experiments confirmed that live *Listeria* can induce low level cytokine and NO production by MyD88-deficient macrophages. To date, only TLR4 has been shown to mediate MyD88-independent signaling events. Therefore, we hypothesize that MyD88-independent responses to live *Listeria* may involve TLR4 by way of surface lipoteichoic acid (12, 18, 19). This point has not yet been tested. It should be noted that the *Listeria* proteins LLO, inlB, plcA, and plcB can stimulate cellular responses through presumably non-TLR-mediated events (44–47). We have ruled out LLO as contributing to the stimulation of MyD88-deficient macrophages by use of live LLO-deficient *Listeria* (data not shown). The contribution of other non-TLR stimulatory molecules cannot be determined at this time.

Our studies surprisingly demonstrate no role for TLR2 or MyD88 in the killing of phagosome-retained *Listeria*; that is to say *Listeria* organisms which are unable to escape to the cytosol either through macrophage activation by IFN- γ or due to the use of LLO-deficient *Listeria*. This indicates that the *Listeria* killing mechanisms present within the vacuolar system do not require any TLR-mediated signals for their induction. We are currently investigating the nature of these listericidal mechanisms.

These results differ from that reported for murine macrophage killing of intracellular *Mycobacterium tuberculosis*, in which TLR2 signals were required for microbicidal activity, via stimulation of NO production (48). However, in this experimental system, a purified exogenous mycobacterial TLR2 ligand (19-kDa lipoprotein) was added to infected cells in addition to TLR2 ligand(s) presumably provided by the infecting bacterium itself. It is noteworthy that in the absence of this additional TLR2 stimulus, no difference was noted between mycobacterial growth in WT vs TLR2-deficient cells. This suggests that live mycobacterial infection itself was an insufficient stimulus for TLR2-induced NO production. It is also important to note that murine macrophage killing of *Listeria* differs from the killing of *M. tuberculosis* in that the former occurs in IFN- γ -activated macrophages in an NO-independent fashion (Fig. 6A and Ref. 26), while the latter requires NO (48).

Our results highlight the fact that in vivo resistance to *Listeria* infection requires MyD88, while in vitro *Listeria* killing by activated macrophages occurs in a MyD88-independent manner. Similar results were reported in iNOS-deficient mice and macrophages (34). In vivo innate immunity to *Listeria* requires a coordinated interplay of cytokines, NO, and cellular responses that require

MyD88 for their appropriate induction. Macrophage listericidal activity is only one component of this response, which when occurring in isolation is insufficient for in vivo resistance. It also remains a possibility that in vivo macrophage *Listeria* killing differs from in vitro macrophage *Listeria* killing, with the former being MyD88-dependent. Studies are underway to address this possibility.

In conclusion, our results demonstrate both the redundancy of TLR-mediated recognition of *Listeria* during in vivo infection, and the absolute requirement for the MyD88 adaptor molecule for in vivo resistance to this pathogen. Surprisingly, we have shown in this study that in vitro macrophage listericidal mechanisms occur in a MyD88-independent manner. These different requirements for MyD88 during in vivo infection vs during the simplified model of in vitro macrophage infection add to our understanding of the innate immune response to *Listeria*, and highlight the need for further investigation into the nature of macrophage listericidal mechanisms.

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