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Mice Lacking Myeloid Differentiation Factor 88 Display Profound Defects in Host Resistance and Immune Responses to *Mycobacterium avium* Infection Not Exhibited by Toll-Like Receptor 2 (TLR2)- and TLR4-Deficient Animals

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To assess the role of Toll-like receptor (TLR) signaling in host resistance to *Mycobacterium avium* infection, mice deficient in the TLR adaptor molecule myeloid differentiation factor 88 (MyD88), as well as TLR2^{-/-} and TLR4^{-/-} animals, were infected with a virulent strain of *M. avium*, and bacterial burdens and immune responses were compared with those in wild-type (WT) animals. MyD88^{-/-} mice failed to control acute and chronic *M. avium* growth and succumbed 9–14 wk postinfection. Infected TLR2^{-/-} mice also showed increased susceptibility, but displayed longer survival and lower bacterial burdens than MyD88^{-/-} animals, while TLR4^{-/-} mice were indistinguishable from their WT counterparts. Histopathological examination of MyD88^{-/-} mice revealed massive destruction of lung tissue not present in WT, TLR2^{-/-}, or TLR4^{-/-} mice. In addition, MyD88^{-/-} and TLR2^{-/-}, but not TLR4^{-/-}, mice displayed marked reductions in hepatic neutrophil infiltration during the first 2 h of infection. Although both MyD88^{-/-} and TLR2^{-/-} macrophages showed profound defects in IL-6, TNF, and IL-12p40 responses to *M. avium* stimulation in vitro, in vivo TNF and IL-12p40 mRNA induction was impaired only in infected MyD88^{-/-} mice. Similarly, MyD88^{-/-} mice displayed a profound defect in IFN- γ response that was not evident in TLR2^{-/-} or TLR4^{-/-} mice or in animals deficient in IL-18. These findings indicate that resistance to mycobacterial infection is regulated by multiple MyD88-dependent signals in addition to those previously attributed to TLR2 or TLR4, and that these undefined elements play a major role in determining bacterial induced proinflammatory as well as IFN- γ responses. *The Journal of Immunology*, 2003, 171: 4758–4764.

Toll-like receptors (TLR)³ play a role in innate defense by recognizing distinct molecular patterns associated with microbial pathogens (1–3). For example, TLR2 is involved in the response to products of Gram-positive bacteria and yeasts (2, 4–6), while TLR4 is required for the recognition of endotoxin of Gram-negative bacteria (4, 7, 8). Stimulation of TLR by microbial products activates NF- κ B and other signaling pathways to produce inflammatory cytokines (2, 9). All TLR share homology with the other members of the IL-1R/TLR superfamily, such as the receptors for IL-1 and IL-18 that also trigger NF- κ B function.

Signal transduction by all of the known TLR, as well as IL-1R/IL-18R, requires an adapter molecule, myeloid differentiation factor 88 (MyD88) (10–12). For this reason, MyD88^{-/-} mice have

been used as a tool for studying the role of TLR in innate and adaptive immunity. MyD88^{-/-} animals fail to generate both proinflammatory and Th1 responses when stimulated with TLR ligands (11–13). These animals are highly susceptible to infection with a wide variety of different pathogens, including *Staphylococcus aureus* (14), *Listeria monocytogenes* (15, 16), and *Toxoplasma gondii* (17), indicating a critical role for MyD88 in host resistance to microbial infection.

Mycobacterium avium is the causative agent of one of the most common opportunistic infections occurring in immunocompromised individuals (18–20). Similar to *Mycobacterium tuberculosis*, *M. avium* stimulates a predominantly cell-mediated immune response, characterized by the production of IFN- γ and other proinflammatory cytokines. This response is thought to play a critical role in host resistance by containing the intracellular growth of the bacteria within macrophages (21, 22). Studies in IFN- γ and IL-12 knockout (KO) mice have confirmed an important function for these cytokines in the control of *M. avium* infection in vivo (23, 24).

To assess the role of the TLR/IL-1R superfamily in induction of host resistance to *M. avium*, we studied the course of infection in animals deficient in MyD88. Because both TLR2 and TLR4 have been previously implicated in the immune control of *M. tuberculosis* (25, 26), we also examined the susceptibility of mice deficient in these specific TLR to *M. avium* challenge. The MyD88^{-/-} animals display dramatically increased susceptibility to *M. avium* infection, a phenotype that correlates with profound defects in neutrophil recruitment, proinflammatory cytokine responses, and CD4⁺ T cell-dependent IFN- γ production. Although not as severe, TLR2-deficient mice also showed impaired host resistance and neutrophil responses to the bacteria. In contrast, infected TLR4

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³ Abbreviations used in this paper: TLR, Toll-like receptor; KO, knockout; MAVAg, soluble *M. avium* Ag; MyD88, myeloid differentiation factor 88; Nrap, natural resistance-associated macrophage protein gene; OSP, outer surface protein; p.i., postinfection; WT, wild type.

KO animals were indistinguishable from WT controls. These findings suggest that in addition to TLR2, host control of *M. avium* infection involves other, as yet to be defined, MyD88-dependent members of the IL-1/TLR superfamily that are specifically required for the generation of the Th1 cytokine response to this pathogen.

Materials and Methods

Mice

Breeding pairs of MyD88^{-/-}, TLR2^{-/-}, and TLR4^{-/-} mice, obtained from Dr. S. Akira (Osaka University, Osaka, Japan) via Dr. D. Golenbock (University of Massachusetts Medical School, Worcester, MA), were used after four to five generations of backcrossing to the C57BL/6 mice. IL-18^{-/-} mice (27) (on C57BL/6 background) were kindly provided by Dr. R. Caspi (National Eye Institute, National Institutes of Health, Bethesda, MD). Mice deficient in IFN- γ (IFN- γ ^{-/-}, on B6 background) or IL-1R β (IL-1RI^{-/-}, on B6129 background) were purchased from The Jackson Laboratory (Bar Harbor, ME). Wild-type (WT) B6/129S1 and C57BL/6 mice were obtained from Taconic (Taconic Farms, Germantown, NY). All mice were maintained at American Association of Laboratory Animal Care-accredited animal facility at the National Institute of Allergy and Infectious Diseases, National Institutes of Health. Mice of both sexes between 8 and 12 wk old were used in all experiments.

Because natural resistance-associated macrophage protein gene (Nramp1) has been shown to influence the resistance of mice to *M. avium* infection (28, 29), we determined the Nramp1 genotype of the partially backcrossed MyD88- and TLR-deficient mice by a published method (30). We found that all three KO lines displayed the Nramp1 susceptibility allele (data not shown), which is expressed by the C57BL/6 parental strain. For this reason, host resistance and immune responses of MyD88 and TLR KO mice to *M. avium* infection were compared with those of C57BL/6 animals, although B6129 mice were also included as controls.

M. avium infection and soluble bacterial Ags

Mice were infected i.v. with 1×10^6 CFU of *M. avium* (strain 2-151-SmT). Bacterial loads in liver and lungs of infected mice were determined at various time points following infection, as previously described (31). Soluble *M. avium* Ags (MAVAg) were also prepared, as described (31).

Tissue cultures and cytokine assays

Single-cell suspensions were prepared from spleens of naive and infected mice. To isolate splenic CD4⁺ T cells, splenocytes were first passed through T cell enrichment columns (R&D Systems, Minneapolis, MN). The enriched T cell populations were then labeled with mAb specific for CD4 and positively selected using magnetic cell sorting (Miltenyi Biotec GmbH, Auburn, CA). Total splenocytes (4×10^6 /ml) were stimulated with medium or MAVAg (20 μ g/ml) for 72 h in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 μ M glutamine, 10 mM HEPES, and 50 μ M 2-ME. IFN- γ levels in culture supernatants were determined by ELISA, as previously described (31). When purified CD4⁺ T cells (1×10^6 /ml) were used, irradiated splenocytes (4×10^6 /ml) from naive animals were added as a source of APC.

Measurement of in vitro macrophage function

Bone marrow cells were cultured for 7 days in complete RPMI supplemented with 20% L929 cell culture supernatant. Adherent macrophages were then plated in triplicates in 48-well plates at 10^5 cells/well and stimulated with 100 U/ml murine IFN- γ in antibiotic-free medium for 16 h. To assess macrophage responses, the cultures were stimulated with a purified TLR2 ligand, *Borrelia burgdorferi* outer surface protein (OSP)-A (5 μ g/ml), or a TLR4 ligand, LPS (100 ng/ml, *Escherichia coli* serotype O55:B5; Sigma-Aldrich, St. Louis, MO). Alternatively, macrophages were infected with live *M. avium* (multiplicity of infection, 10:1) for 4 h; the adherent cells were washed twice; and fresh complete RPMI was added. Culture supernatants were collected 72 h after stimulation or infection, and TNF- α , IL-6, and IL-12 p40 levels were assessed by ELISA using commercial kits (R&D Systems) or previously published assays (31). Following removal of supernatants, *M. avium*-infected macrophages were lysed using 1% saponin. The cell lysates were cultured on 7H11 agar to determine bacterial CFU.

Real-time RT-PCR measurement of cytokine mRNA

Liver tissue was homogenized in 1 ml of TRIzol (Invitrogen, San Diego, CA). Total RNA was extracted according to the manufacturer's directions with an additional chloroform extraction. One microgram of total RNA was reverse transcribed using Super Script II RNase H⁻ reverse transcriptase (Invitrogen). The cDNA was then subjected to real-time RT-PCR using SYBR Green (Applied Biosystems, Foster City, CA) on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). The following primer pairs were used: hypoxanthine phosphoribosyltransferase, GTTG GATACAGGCCAGACTTTGTTG (F) and GATTCAACTGCGCT CATCTTAGGC (R); IFN- γ , GGCCATCAGCAACAACATAAGC (F) and AATCTGAGTTCAGTCAGCCGCT (R); TNF- α , TTCTGTCTACT GAACTTCGGGGTG (F) and GGAAGACTCTCCAGGTATATGG (R); IL-12p40, CTCACATCTGCTGCCACAAG (F) and AATTTGGT GCTTCACACTTCAGG (R). Each sample was normalized to hypoxanthine phosphoribosyltransferase, and gene expression relative to the level in uninfected controls was calculated.

Analysis of neutrophil recruitment

Total hepatic leukocytes were isolated by the method of Bertolino et al. (32). Briefly, livers were perfused with PBS to remove blood leukocytes. A single-cell suspension was prepared by sieving liver lobes through a 100- μ m-pore-size mesh. Liver leukocytes were enriched by Percoll gradient centrifugation and stained with mAb specific for Gr-1, CD11b, and I/A (BD Biosciences, San Diego, CA). The percentage of granulocytes (Gr-1⁺CD11b⁺I/A⁻) among hepatic leukocytes was determined by flow cytometry using a FACSCalibur (BD Immunocytometry System, San Jose, CA). Data were collected and analyzed with the CellQuest program.

Histopathology

Tissue sections from livers and lungs were fixed with Formalin, sectioned, and stained with H&E. The Ziehl-Neelsen method was used to stain acid-fast mycobacteria in tissue sections.

Statistics

ANOVA was used to analyze the significance of differences in means between multiple experimental groups. The multicomparison significance level for the one-way ANOVA was 0.05. If significance was detected by one-way analysis, pair-wise differences were evaluated using Fisher's protected least significant difference ANOVA post hoc test. Statistical significance was defined as $p < 0.05$.

Results

MyD88^{-/-} mice fail to control *M. avium* replication and succumb to the infection

To investigate the role of TLR/MyD88 signaling in host defense against mycobacterial infection in vivo, MyD88^{-/-}, TLR2^{-/-}, TLR4^{-/-}, and WT control C57BL/6 and B6/129 animals were infected i.v. with *M. avium* (10^6 CFU). Survival of animals and hepatic and pulmonary bacterial loads was compared at various time points thereafter. All MyD88^{-/-} mice succumbed between 9 and 14 wk postinfection (p.i.), whereas the WT B6 or B6129 (data not shown), TLR2^{-/-}, and TLR4^{-/-} animals showed 100% survival during the same period. By 16–22 wk, however, TLR2 mice also succumbed to the infection. In contrast, the majority of TLR4 animals survived for the 25-wk course of the experiment (Fig. 1).

The accelerated mortality of infected MyD88^{-/-} mice was associated with uncontrolled mycobacterial proliferation in the liver and lungs. When bacterial burdens were assayed in liver, MyD88^{-/-} mice showed 1–1.5 log₁₀ increases in CFU compared with Nramp1 coisogenic C57BL/6 WT controls at 2 and 6 wk postinfection (Fig. 2). An even larger increase in bacterial counts was evident in the lungs of the same MyD88 KO animals at wk 6 following mycobacterial exposure.

Because both TLR2 and TLR4 have been implicated in host resistance to mycobacterial infection (25, 26), we also evaluated bacterial burdens in mice deficient for these two MyD88-associated TLR. Hepatic bacterial burdens were significantly elevated in TLR2^{-/-} relative to WT controls at both 2 and 6 wk, while

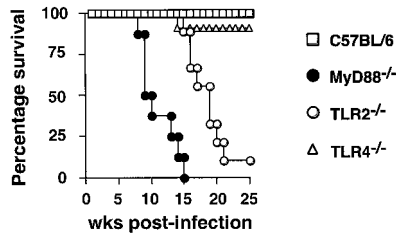


FIGURE 1. Infected MyD88^{-/-} mice display increased mortality relative to both TLR-deficient and WT animals. Survival of MyD88^{-/-}, TLR2^{-/-}, TLR4^{-/-}, and WT C57BL/6 mice ($n = 10\text{--}15/\text{group}$) was monitored following i.v. *M. avium* infection. Data are pooled results from two separate experiments that gave nearly identical results.

TLR4^{-/-} mice showed no significant increases. A similar elevation in CFU was observed in the lungs of TLR2, but not TLR4, KO mice at wk 6 p.i. This difference in host resistance between TLR2^{-/-} and TLR4^{-/-} animals was maintained into the chronic phase of infection. At 10 wk p.i., bacterial CFU in TLR2^{-/-} mice ($\log_{10} 8.8 \pm 0.5$ and 6.9 ± 0.2 in liver and lung, respectively) were significantly higher ($p < 0.05$) than those measured in either WT ($\log_{10} 7.8 \pm 0.2$ and 5.8 ± 0.1) or TLR4^{-/-} ($\log_{10} 7.9 \pm 0.1$ and 5.6 ± 0.1) animals. Accurate bacterial burdens could not be determined on chronically infected MyD88^{-/-} mice because of their mortality during this period. Nevertheless, at each of the earlier time points examined, the MyD88^{-/-} mice showed higher bacterial loads than TLR2^{-/-} animals (Fig. 2). The latter difference was particularly striking when CFU were measured in lung. Interestingly, bacterial burdens in the lungs of MyD88 KO mice exceeded even those of IFN- γ KO mice, although no difference was observed in the liver.

In addition to TLR signaling, MyD88 also controls IL-1R- and IL-18R-dependent cellular activation (11). To examine the possible role of these cytokine receptors in the effects of MyD88 deficiency on *M. avium* infection, bacterial burdens were also examined in IL-18^{-/-} mice as well as mice deficient in IL-1R. No significant differences in CFU were evident in lung or liver of IL-1R^{-/-} or IL-18^{-/-} mice in comparison with their background-matched controls.

MyD88^{-/-} mice display impaired granuloma function and exacerbated pulmonary pathology

Because the granulomatous response plays a critical role in controlling mycobacterial dissemination, we next examined whether granuloma formation and bacterial replication within these tissue lesions differ between infected WT and KO mice. Hepatic granulomas of MyD88 and TLR KO mice did not show any apparent changes in leukocyte composition at wk 2 and 6 p.i. (data not shown). The size of hepatic granulomas appeared to be similar in all mouse strains (Fig. 3, A–D), and this was confirmed by direct measurement of mean lesion diameters (WT = $57.1 \pm 5.9 \mu\text{m}$, MyD88^{-/-} = $64.6 \pm 5.2 \mu\text{m}$, TLR4^{-/-} = $61.6 \pm 5.2 \mu\text{m}$, TLR2^{-/-} = $69.0 \pm 6.7 \mu\text{m}$, $n = 4$). No tissue necrosis was evident in the hepatic tissue sections of any of the mouse strains examined. Bacilli were largely confined within granulomas in both WT and MyD88^{-/-} animals. However, in contrast to the hepatic granulomas in WT mice (Fig. 3E), in which only a few bacteria were detected by acid-fast staining, those in MyD88^{-/-} mice contained large numbers of bacilli (Fig. 3F).

Histological examination of lungs in infected MyD88^{-/-} (Fig. 3, H and L) mice revealed dramatic pathological changes not evident in WT (Fig. 3, G and K), TLR2^{-/-} (Fig. 3J), TLR4^{-/-} (Fig. 3I), or IFN- γ ^{-/-} or IL-18^{-/-} mice (data not shown). Small gran-

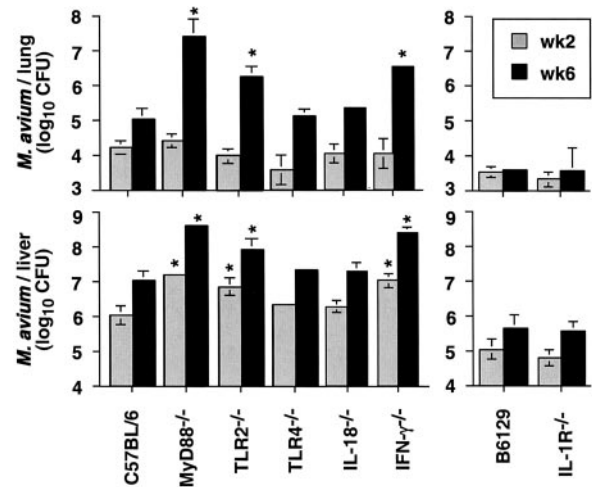


FIGURE 2. MyD88^{-/-} mice show significantly impaired control of *M. avium* growth. Hepatic and pulmonary bacterial burdens (CFU) in WT and KO mice were compared at wk 2 (gray bar) and 6 (filled bar) following i.v. infection with *M. avium*. The means (\pm SD) of three to four mice per time point are shown. The significance of the differences in CFU between WT and KO strains was compared by ANOVA (*, $p < 0.05$). The experiment shown is representative of two performed.

ulomas that contained approximately equal numbers of macrophages and lymphocytes developed in the lungs of WT, TLR2^{-/-}, and TLR4^{-/-} mice. In contrast, pulmonary granulomas were much larger in MyD88^{-/-} animals and formed destructive lesions composed mainly of necrotic macrophages and lymphocytes that occupied ~40% of the lung area (Fig. 3, H and L).

Impaired neutrophil recruitment in *M. avium*-infected MyD88^{-/-} and TLR2^{-/-} mice

It has been shown previously that following i.v. inoculation, bacteria are taken up by Kupffer cells in the liver, where they provoke a rapid neutrophil response that is thought to play a role in reducing early pathogen loads (33, 34). For this reason, we asked whether a defect in early neutrophil recruitment might underlie the increased susceptibility of either MyD88- or TLR2-deficient mice to *M. avium* infection. To perform this analysis, livers were collected from mice 2 h following i.v. bacterial inoculation (5×10^6 CFU per animal), and hepatic Gr-1⁺CD11b⁺ neutrophils were quantitated by flow cytometry. In agreement with published data on other bacterial infection models (33, 34), *M. avium* infection led to a significant increase in the percentage as well as absolute numbers of Gr-1⁺CD11b⁺ (I/A⁻, data not shown) cells in the livers of WT and TLR4^{-/-} mice compared with uninfected animals (Fig. 4, A and B, and data not shown). In contrast, infected MyD88^{-/-} and TLR2^{-/-} mice failed to show a major increase in either the number or the percentage of neutrophils when examined at the same time point.

MyD88 is required for the proinflammatory cytokine response to *M. avium* infection

MyD88 regulates the induction of proinflammatory cytokines by microbial products from macrophages and dendritic cells. To assess whether the enhanced susceptibility of MyD88^{-/-} mice to *M. avium* infection is associated with an impaired cytokine production, we compared IL-6, TNF, and IL-12p40 responses of bone marrow-derived macrophage from KO and WT animals with *M. avium* infection in vitro. Upon stimulation with TLR agonists (OSP-A and LPS for TLR2 and TLR4, respectively) or infection

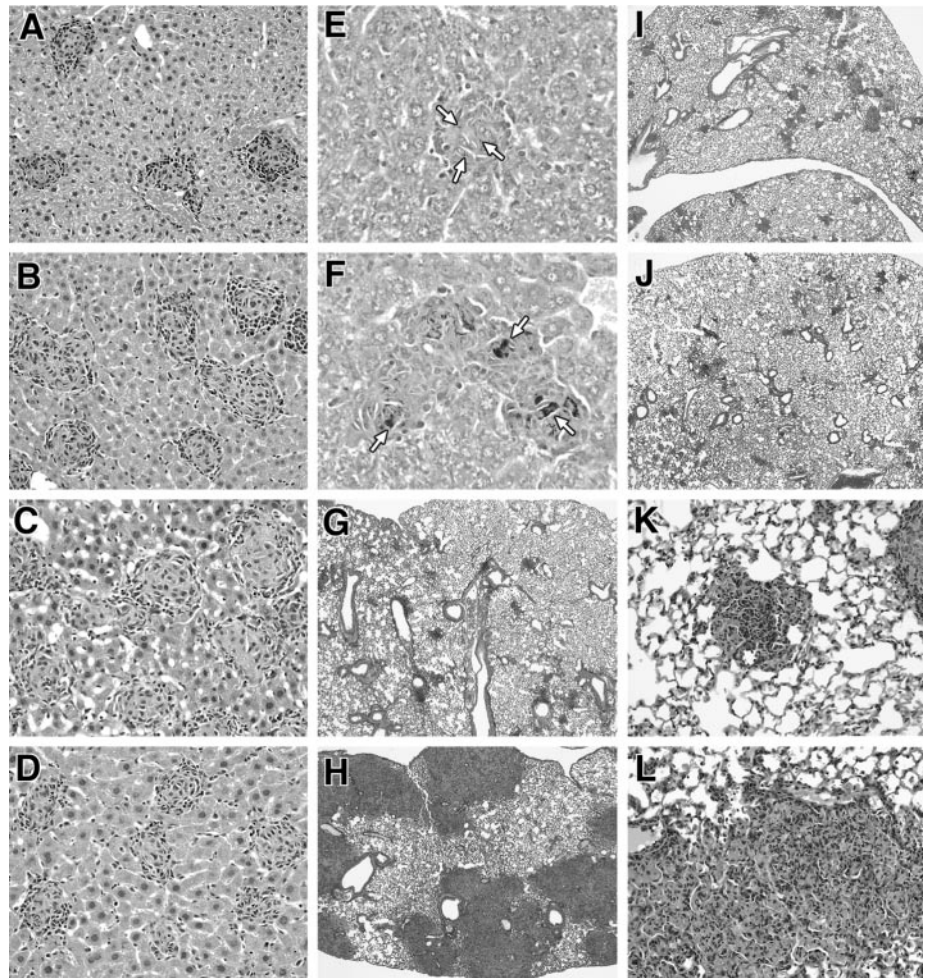


FIGURE 3. MyD88^{-/-} mice show impaired bacterial clearance within hepatic granulomas and exacerbated pulmonary pathology. Formalin-fixed, paraffin-embedded hepatic (A–F) and pulmonary (G–L) tissue sections from 6-wk infected mice were stained with H&E. Acid-fast bacilli in hepatic granulomas were identified with the Ziehl-Neelsen method (E and F). Positively stained *M. avium* bacilli in hepatic sections are indicated by arrows. The representative sections shown are from infected WT (A, E, G, and K), MyD88^{-/-} (B, F, H, and L), TLR2^{-/-} (C and I), and TLR4^{-/-} (D and J) mice. Magnification shown is $\times 200$, except for K and L, which are $\times 600$.

with live *M. avium*, macrophages from WT mice produced high levels of IL-6, TNF, and IL-12p40 (Fig. 5A). In contrast, MyD88^{-/-} macrophages failed to produce the same cytokines in response to either *M. avium* infection or TLR2 or TLR4 ligand stimulation. As expected, TLR2^{-/-} macrophages responded to stimulation with LPS, but not OSP-A, and produced reduced levels of the three proinflammatory cytokines when exposed to *M. avium*. In contrast, *M. avium*-stimulated TLR4^{-/-} macrophage displayed unimpaired inflammatory cytokine responses comparable to WT cells, confirming that recognition of *M. avium* products in vitro is mediated largely by TLR2, as previously reported (6, 35). In the same experiments, we also compared levels of intracellular infection of *M. avium*-exposed, IFN- γ -primed macrophages at 72 h p.i. by measuring CFU in cell lysates. We found no major differences in bacterial counts in the WT, MyD88^{-/-}, TLR2^{-/-}, and TLR4^{-/-} macrophage cultures during this period (data not shown). Consistent with the in vitro findings concerning proinflammatory cytokine production, in vivo TNF and IL-12p40 mRNA levels in livers were significantly reduced in 2-wk infected MyD88^{-/-} as measured by RT-PCR. However, the levels of the same cytokines in the livers of infected TLR2^{-/-} mice were comparable to those in TLR4^{-/-} and WT animals (Fig. 5B).

M. avium-infected MyD88^{-/-} mice display impaired IFN- γ responses

It is known that mycobacterial infection stimulates a strong Th1 response associated with production of IFN- γ . This adaptive immune response is essential for containing bacterial growth within

granulomas in vivo. To investigate whether MyD88^{-/-} mice generate a normal Th1 response following *M. avium* infection, splenic CD4⁺ T cells purified from 2-wk infected mice were stimulated with MAVAg in the presence of naive splenocytes from WT animals and secreted IFN- γ measured 72 h later. As shown in Fig. 6A, production of IFN- γ by CD4⁺ T cells of MyD88^{-/-} mice was impaired compared with WT as well as TLR2- and TLR4-deficient animals. The diminished IFN- γ production in infected MyD88^{-/-} mice was not due to the induction of a Th2 response in these animals because neither IL-4 nor IL-5 was detected in the same cultures (data not shown).

To confirm that the impairment in IFN- γ production observed ex vivo also occurs in vivo, we measured mRNA levels for the cytokine in the livers of KO and WT mice (Fig. 6B). At 2 wk p.i., IFN- γ mRNA levels in the infected MyD88^{-/-} mice were ~ 30 -fold lower relative to WT controls. In contrast, IFN- γ mRNA levels in livers of infected TLR2- and TLR4-deficient animals were not significantly different from WT control levels.

Because it was possible that the defect in IFN- γ production in 2-wk infected MyD88^{-/-} merely reflects a delay in this cytokine response, we measured MAVAg-induced IFN- γ synthesis by spleen cells over a 4-wk interval following infection. As shown in Fig. 6C, spleen cells from MyD88^{-/-} mice showed severely impaired IFN- γ production throughout the entire course of this experiment.

Because the impairment in *M. avium*-specific IFN- γ production in MyD88 KO mice could also be a result of the absence of IL-18R signaling (36), we examined the possible contribution of IL-18 in

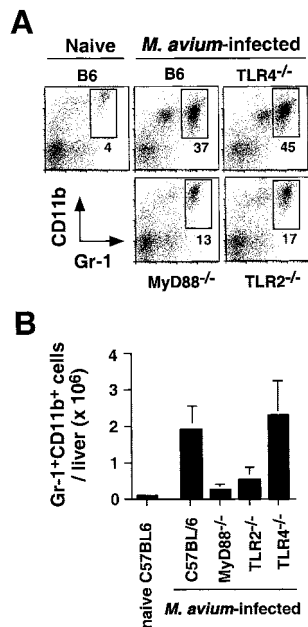


FIGURE 4. MyD88^{-/-} and TLR2^{-/-} mice display impaired hepatic neutrophil influx during acute *M. avium* infection. Leukocytes isolated from livers of WT, MyD88^{-/-}, TLR2^{-/-}, and TLR4^{-/-} animals at 2 h p.i.; stained with anti-Gr-1, CD11b, and I/A mAb; and analyzed by flow cytometry. The percentages (A) and absolute numbers (B) of Gr-1⁺CD11b⁺ leukocytes are shown. The mean (\pm SD) of three individual mice is shown. The experiment shown is representative of two performed.

IFN- γ responses to *M. avium* infection. When purified CD4⁺ T cells from 2-wk infected mice were restimulated with MAVAg in vitro, CD4⁺ T cells from IL-18^{-/-} and WT mice produced similar levels of IFN- γ (WT vs IL-18^{-/-} mice, 6.48 ± 0.1 and 7.81 ± 0.45 ng/ml, respectively). Similarly, mRNA levels of IFN- γ in the livers of the IL-18^{-/-} mice were comparable to those of WT animals (data not shown).

Discussion

Although TLR signaling has been implicated in the host response to mycobacteria, most of the evidence has been obtained from in vitro studies in which both TLR2 and TLR4 have been shown to regulate cytokine as well as NF- κ B activation following stimulation with either live bacteria or mycobacterial products (6, 35, 37, 38). In different reports, these two TLR family members have also been shown to have significant, but partial effects on control of *M. tuberculosis* infection in mice (25, 26). In the present study, we have compared host resistance and immune responses to *M. avium* in MyD88^{-/-}, TLR2^{-/-}, and TLR4^{-/-} animals with the goal of assessing the relative contribution of these as well as additional TLR in mycobacterial immunity.

We observed that MyD88^{-/-} mice are significantly more susceptible to *M. avium* infection than either of the two TLR-deficient strains. Moreover, in agreement with one recent study with *M. tuberculosis* (26), we found that TLR4^{-/-} mice are indistinguishable from WT controls in both bacterial burden and immune responses. This finding is also consistent with previously published in vitro observations, indicating that *M. avium* stimulates human TLR2, but not TLR4 function (6, 35). The difference in bacterial load between MyD88^{-/-} and TLR2^{-/-} mice was most pronounced in the lung at 6 wk postinfection, at which time point massive necrotic lesions were evident throughout the lung that were not present in WT, TLR2^{-/-}, TLR4^{-/-}, IL-18^{-/-}, or IFN- γ ^{-/-} animals. Similar destructive lesions were observed in livers

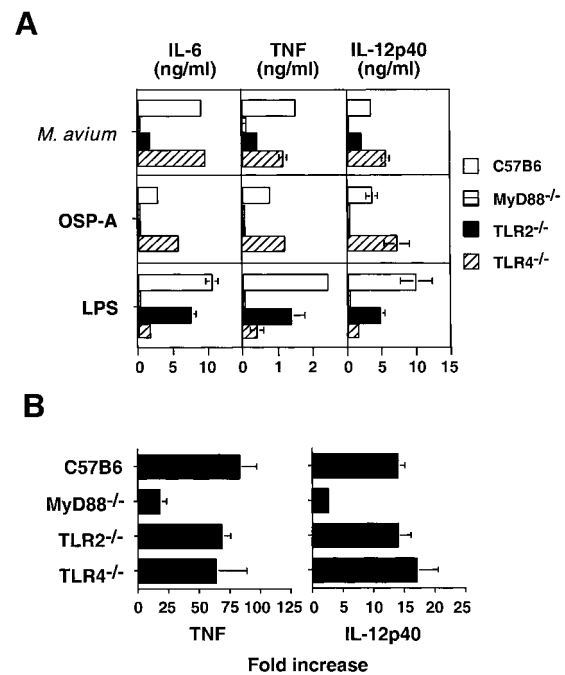


FIGURE 5. MyD88 is essential for proinflammatory cytokine responses to *M. avium* infection. A, MyD88^{-/-} and TLR2^{-/-} macrophages show diminished production of IL-6, TNF, and IL-12p40 in response to *M. avium* infection in vitro. Bone marrow-derived macrophages from WT and KO mice were stimulated with OSP-A, LPS, or live *M. avium*. Secreted IL-6, TNF, and IL-12p40 were determined 72 h later by ELISA. The data shown are the means (\pm SD) of triplicate cultures. B, TNF and IL-12p40 mRNA expression is impaired in the livers of 2-wk infected MyD88^{-/-}, but not TLR2^{-/-} or TLR4^{-/-} mice. Cytokine mRNA levels were determined by real-time PCR and expressed as fold increase compared with the baseline levels of uninfected animals. The mean (\pm SD) of three to four individual mice is shown. Data shown are representative of two separate experiments performed.

of *M. avium*-infected TNFRp55^{-/-} mice by Ehlers et al. (39), raising the possibility that the pathology occurring in infected MyD88^{-/-} mice may be a consequence of the impaired TNF production seen in the latter animals. Nevertheless, in contrast with the previous findings in TNFRp55^{-/-} mice, we failed to observe significant tissue destruction in liver sections from *M. avium*-infected MyD88^{-/-} animals. The latter finding suggests that MyD88-dependent effector mechanisms are of greater importance in the control of bacterial growth in the lung, as opposed to liver. This observation may relate to differences in effector cell populations in the lung as compared with other organs.

M. avium-infected MyD88^{-/-} mice also showed greater defects in mycobacterium-induced immune responses than any of the other gene-deficient animals examined in this study, although some of these defects were shared with other deficient mouse strains. The most profound immune response impairments were seen in the proinflammatory and Th1-associated cytokine responses mounted by MyD88^{-/-} animals. Thus, while bone marrow-derived macrophages from both MyD88^{-/-} and TLR2^{-/-} mice were defective in their production of IL-6, IL-12p40, and TNF in vitro, only MyD88^{-/-} mice showed impaired IL-12p40 and TNF mRNA expression in vivo. Similarly, MyD88^{-/-}, but not TLR2^{-/-}, mice showed reduced IFN- γ production by CD4⁺ T cells ex vivo or diminished IFN- γ mRNA levels in liver in situ. Importantly, *M. avium*-infected IL-18^{-/-} mice failed to show the

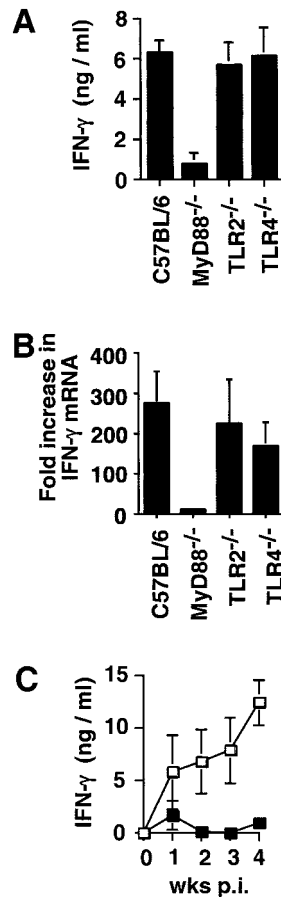


FIGURE 6. *M. avium*-induced IFN- γ production is impaired in MyD88^{-/-}, but not TLR2^{-/-} or TLR4^{-/-}, mice. **A**, Purified splenic CD4⁺ T cells from 2-wk infected WT, MyD88^{-/-}, TLR2^{-/-}, and TLR4^{-/-} mice were restimulated with MAVAg for 72 h. IFN- γ in the culture supernatants was then measured by ELISA. **B**, Expression levels of IFN- γ mRNA in the livers of 2-wk infected animals were determined by real-time PCR and presented as fold increase compared with that in uninfected animals. The mean (\pm SD) from three to four individual mice is shown. **C**, Splenocytes isolated at weekly intervals from infected WT (B6129, open symbol) and MyD88^{-/-} (filled symbol) mice were restimulated with MAVAg and secreted IFN- γ measured 72 h later by ELISA. The mean (\pm SD) of data points from three to four individual mice is shown. The experiment shown in each panel is representative of two performed.

same defects in IFN- γ response, arguing that the observed impairment in *M. avium*-specific IFN- γ production in MyD88 KO mice is not a result of the absence of IL-18R signaling.

Although infected MyD88^{-/-} mice showed unique defects in cytokine production, they shared with TLR2^{-/-} animals a major defect in early recruitment of neutrophils to the liver. Pathogens that transit through the blood during acute infection are subject to hepatic clearance mediated jointly by resident tissue macrophages, Kupffer cells, and neutrophils recruited to the liver in response to microbial stimulation (33, 34). In the case of *M. avium* (40, 41), *M. tuberculosis* (42), and BCG (43) infections, depletion of neutrophils results in an early increase in bacterial growth, suggesting an important role for these host cells in innate defense against mycobacteria. Nevertheless, the nature of the pattern recognition events responsible for the induction of neutrophil recruitment by bacteria is poorly understood. Our observation of defective influx of neutrophils into livers of acutely infected MyD88- and TLR2-deficient mice indicates that TLR signaling plays an important role

in triggering this cellular event and is in agreement with similar observations made in a model of *Listeria* infection (15).

Although the observed defect in neutrophil recruitment in MyD88- and TLR2-deficient mice may result from impaired chemokine production, preliminary experiments failed to reveal significant differences in the expression of mRNAs for the neutrophil chemotactic chemokines, KC and macrophage-inflammatory protein-2, in livers of WT and KO animals at 2 and 6 h p.i. (data not shown). An alternative possibility is that the impaired neutrophil migration in infected MyD88^{-/-} and TLR2^{-/-} animals results from a defect in the response of the neutrophils themselves to mycobacterial stimulation. In this regard, the 19-kDa mycobacterial lipoprotein, a TLR2 ligand, has been shown to directly activate neutrophil functions (44). Nevertheless, it remains to be determined whether the response to this or other bacterial derived TLR2 ligands triggers neutrophil migration in vivo or whether the absence of early neutrophil accumulation is a contributing factor in the impaired control of *M. avium* infection in TLR2^{-/-} and MyD88^{-/-} animals.

An important conclusion of the current study is that while MyD88 is absolutely required, TLR2 is dispensable for the generation of some antimycobacterial effector mechanisms. This is particularly true for the responses measured in vivo or ex vivo (e.g., levels of IFN- γ , IL-12p40, and TNF), in which infected TLR2^{-/-} mice were indistinguishable from WT animals. Because TLR2^{-/-} macrophages are nevertheless defective in their response to *M. avium* in vitro, one possibility is that the in vivo loss of immune function due to TLR2 deficiency is compensated efficiently by other MyD88-dependent signaling pathways, such as those triggered by IL-1 and IL-18. Moreover, tissue pathology resulting from mycobacterial infection may also contribute to MyD88-dependent cellular activation via a TLR4-dependent pathway, as demonstrated by recent studies showing that heat shock proteins (45, 46), hyaluronan (47), and fibronectins (48) released from damaged cells can trigger TLR4 signaling. Whether such MyD88-dependent functions become overexpressed in infected TLR2^{-/-} mice, thereby compensating for the loss in TLR2 function, remains to be determined.

Alternatively, the marked susceptibility to *M. avium* infection of MyD88^{-/-} relative to TLR2^{-/-} mice may reflect the requirement for multiple TLR, including TLR2, in host resistance to mycobacterial infection. Such TLR may include TLR9, which is likely to be triggered by CpG oligonucleotides presented in mycobacterial DNA (49–51), or TLR4, which although not required in itself for control of *M. avium* infection in our experiments, may nevertheless play a synergistic role with TLR2 in generating the appropriate effector response. A final possibility is that the enhanced susceptibility of infected MyD88^{-/-} mice is due to the involvement of as yet to be identified pathway(s) requiring the MyD88 adaptor molecule. Studies in mice with combined deficiency in multiple TLR should help in distinguishing between these alternative explanations of the relative contributions of MyD88 and TLR2 in host control of mycobacterial infection.

The findings presented in this work underscore the major role played by innate signaling mechanisms in host resistance to mycobacteria. Further delineation of these pathways may lead to important information concerning determinants of host susceptibility to mycobacterial diseases in humans as well as to improved strategies for antimycobacterial immunization.

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