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Toll-Like Receptor 9 Is Required for Full Host Resistance to *Mycobacterium avium* Infection but Plays No Role in Induction of Th1 Responses[▽]

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To investigate the role of Toll-like receptor 9 (TLR9) in innate immunity to *Mycobacterium avium*, TLR9, TLR2, and MyD88 knockout (KO) mice were infected with this bacterium. Bacterial burdens were higher in the spleens, livers, and lungs of infected TLR9 KO mice than in those of C57BL/6 mice, indicating that TLR9 is required for efficient control of *M. avium* infection. However, TLR9 KO or TLR2 KO spleen cells displayed normal *M. avium*-induced tumor necrosis factor alpha (TNF-α) and gamma interferon (IFN-γ) responses. This finding was confirmed by determining the number of splenic $CD4^+$ T cells producing IFN-γ by flow cytometry. Furthermore, TLR2 and MyD88, but not TLR9, played a major role in interleukin-12 and TNF-α production by *M. avium*-infected macrophages and dendritic cells (DCs). We also found that major histocompatibility complex class II molecule expression on DCs is regulated by TLR2 and MyD88 signaling but not by TLR9. Finally, lack of TLR9, TLR2, or MyD88 reduced the numbers of macrophages, epithelioid cells, and lymphocytes in *M. avium*-induced granulomas but only MyD88 deficiency affected the number of liver granulomas. In summary, our data demonstrated that the involvement of TLR9 in the control of *M. avium* infection is not related to the induction of Th1 responses.

Mycobacterium avium is an opportunistic pathogen that infects mostly immunocompromised individuals (15). After entering the host, mycobacteria are internalized by macrophages and survive host killing by preventing phagolysosome fusion, inhibiting the acidification of the phagocytic vacuole, and disrupting the actin cytoskeleton of the cell (1). Similarly to infection with *Mycobacterium tuberculosis*, the interaction of *M. avium* with phagocytic cells results in the recruitment of $CD4^+$ T lymphocytes and in the secretion of gamma interferon (IFN-γ) and other proinflammatory cytokines (5). This process is suggested to be at least partially dependent on the recognition of pathogen-associated molecular patterns by Toll-like receptors (TLRs) expressed on innate immunity cells, which in turn leads to MyD88-mediated activation of the cytokine gene-associated transcription factor NF-κB (19, 20, 24).

The involvement of TLRs in host resistance to *M. avium* was suspected given the greatly augmented susceptibility of MyD88-deficient mice to infection with this bacterium (6, 10). Even though TLR2 has been proposed to be a major receptor for the recognition of glycopeptidolipids from *M. avium* (28), TLR2 knockout (KO) mice were found to be less susceptible to *M. avium* infection than MyD88 KO mice. Additionally, it has been reported that TLR4 deficiency does not affect the resistance of mice to *M. avium* (6). Therefore, these findings suggest that, in addition to TLR2, host control of *M. avium* infection involves other, as-yet-undefined, MyD88-dependent members of the interleukin-1 (IL-1)/TLR superfamily.

In the present study, we investigated the role of TLR9 in innate immune recognition of *M. avium*, since this receptor localizes to the endosomes and phagolysosomes, where it may be triggered by mycobacterial CpG DNA motifs upon uptake of the pathogen. It is worth noting that it has already been demonstrated that TLR9 recognition modulates the immune response to *M. tuberculosis* and cooperates with TLR2 in mediating optimal resistance to this bacterium (2).

Herein, we have observed that TLR9 is required for efficient control of *M. avium* infection in mice, even though it does not seem to be necessary for full production of the proinflammatory cytokines IFN-γ and tumor necrosis factor alpha (TNF-α) by splenic cells *in vitro*. Furthermore, our findings corroborate the already described roles of TLR2 and MyD88 in the host response to *M. avium* infection and extend this previous knowledge by demonstrating that TLR2, but not TLR9, is involved in the expression of major histocompatibility complex (MHC) class II molecules and in the production of IL-12 and TNF-α by *in vitro*-stimulated dendritic cells (DCs) and macrophages.

MATERIALS AND METHODS

Experimental animals. MyD88 KO, TLR2 KO, and TLR9 KO mice from breeding pairs provided by S. Akira (Osaka University, Osaka, Japan) were maintained in our animal facilities. C57BL/6 wild-type mice were obtained from the Federal University of Minas Gerais (UFMG, Belo Horizonte, Brazil). *Nramp1* genotyping was performed according to Medina et al. (23) in order to

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confirm the susceptibility of the different mouse lineages to *M. avium* infection (data not shown).

Bacterial strains and growth conditions. Low-virulence *M. avium* strains 2447 and 2-151, exhibiting a smooth transparent morphotype (SmT) (10), were grown in Middlebrook 7H9 broth containing 0.05% Tween 80 and 0.2% glycerol and supplemented with 10% albumin-dextrose-catalase (ADC). Mid-exponential-phase cultures were harvested by centrifugation, resuspended in saline with 0.05% Tween 80, briefly sonicated, and stored at −70°C until used.

***In vivo* infection with *M. avium* and measurement of bacterial loads in mouse organs.** Eight- to 12-week-old mice were infected intravenously with 1×10^6 CFU of *M. avium* strain 2447. The bacterial loads in the spleens, livers, and lungs of infected mice were determined at 30 and 100 days after infection. Briefly, the organs were aseptically collected and homogenized in distilled water containing 0.05% Tween 80. Serial dilutions of the resulting suspensions were plated in Middlebrook 7H10 agar medium supplemented with 10% oleic acid-ADC (OADC), and the CFU were counted following incubation for 8 to 10 days at 37°C.

***In vitro* stimulation of mouse splenic cells.** Cells obtained from the spleens of infected mice were washed with saline, and the erythrocytes were lysed using a hemolytic solution (155 mM NH_4Cl, 10 mM $KHCO_3$, pH 7.2). Splenic cells were seeded at 10^6/well into wells of 96-well plates containing RPMI 1640 (Gibco, Carlsbad, CA) supplemented with 2 mM L-glutamine, 25 mM HEPES, 10% heat-inactivated fetal bovine serum (FBS; Gibco, Carlsbad, CA), penicillin G sodium (100 U/ml), and streptomycin sulfate (100 μg/ml). The cells were stimulated with *M. avium* 2447 (multiplicity of infection [MOI], 5:1), Pam3CSK4 (a TLR2 ligand; 1 μg/ml; InvivoGen, San Diego, CA), CpG ODN1826 (a TLR9 ligand; 1 μg/ml; InvivoGen), or *Escherichia coli* lipopolysaccharide (LPS; 1 μg/ml; Sigma). Unstimulated cells were used as a negative control, and cells stimulated with concanavalin A (ConA; 5 μg/ml) were the positive control for T-cell activation. After 48 and 72 h of culture, respectively, the levels of TNF-α and IFN-γ in cell supernatants were measured by enzyme-linked immunosorbent assay (ELISA) using the Duoset kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Generation and *in vitro* stimulation of bone marrow (BM)-derived macrophages (BMMs) and DCs (BMDCs). Macrophages were derived from BM of wild-type, TLR9 KO, TLR2 KO, and MyD88 KO mice as previously described (12). Briefly, BM cells were removed from the femurs and tibias of the animals and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA) containing 10% FBS (HyClone, Logan, UT), 1% HEPES, and 10% L929 cell-conditioned medium (LCCM), as a source of monocyte colony-stimulating factor (M-CSF), in 24-well plates (5×10^5 cells/well). After 4 days, 100 μl/well LCCM was added. At day 7, the medium was renewed. At day 10 of culture, when the cells had completely differentiated into macrophages, the medium was harvested and we added supplemented DMEM (500 μl/well) containing *M. avium* 2447 (MOI, 5:1), Pam3CSK4 (1 μg/ml; InvivoGen, San Diego, CA), CpG ODN1826 (1 μg/ml; InvivoGen), or *E. coli* LPS (1 μg/ml; Sigma, St. Louis, MO). BMMs from C57BL/6 and TLR9 KO mice were also stimulated with *M. avium* genomic DNA (10 μg/ml). DCs were derived from the femurs and tibias of wild-type, TLR9 KO, TLR2 KO, and MyD88 KO mice as described by Macedo et al. (21). Briefly, BM cells were cultured in DMEM (Gibco, Carlsbad, CA) containing 10% FBS (HyClone, Logan, UT), 100 U/ml penicillin, and 100 μg/ml streptomycin plus 20 ng/ml murine recombinant granulocyte-M-CSF (GM-CSF). Petri dishes containing 1×10^7 cells were incubated at 37°C in an atmosphere of 5% CO_2. At day 3 of incubation, a further 5 ml of fresh complete medium containing GM-CSF was added, and on days 6 and 8, 3 ml of medium was removed from the culture and replaced with fresh supplemented medium containing GM-CSF. At day 10, nonadherent cells were harvested and used to seed round-bottom 96-well culture plates (3×10^5 cells/well). Stimulation of the BMDCs followed the same protocol described above for BMMs, but the stimuli were added in 200 μl DMEM/well. Culture supernatants of BMMs and BMDCs were collected after 24 h of stimulation and assayed for the concentrations of IL-12p40 and TNF-α by ELISA (R&D Systems).

Infection of BMMs. At day 10 of culture, the macrophages were infected with *M. avium* 2447 (MOI, 2:1) in 200 μl/well DMEM (Gibco, Carlsbad, CA) containing 10% FBS (HyClone, Logan, UT), 1% HEPES, and 10% LCCM. Cells were incubated for 4 h at 37°C in a 5% CO_2 atmosphere, washed with warm Hanks balanced salt solution (Gibco, Carlsbad, CA) to remove noninternalized bacteria, and then reincubated in supplemented DMEM. To quantify the number of intracellular bacteria, macrophages from triplicate wells were immediately lysed with 0.1% saponin (Sigma, St. Louis, MO). Serial dilutions were plated in Middlebrook 7H10 agar medium supplemented with 10% OADC, and the CFU were counted after 8 to 10 days of incubation. Other cells were incubated for 4 or 7 days to measure the intracellular growth of bacteria.

Real-time reverse transcription (RT)-PCR. BMMs from C57BL/6, TLR2 KO, TLR9 KO, and MyD88 KO mice were infected with *M. avium* strain 2447 or stimulated with Pam3CSK4 or CpG ODN1826. The treated cells were homogenized in TRIzol reagent (Invitrogen) to isolate total RNA. RT of 1 μg of total RNA was performed using the Enhanced Avian RT-PCR kit (Sigma, St. Louis, MO) according to the manufacturer's instructions. Real-time RT-PCR was conducted with a final volume of 10 μl containing SYBR green PCR Master Mix (Applied Biosystems, Carlsbad, CA), oligo(dT) cDNA as the PCR template, and 20 μM primers. Real-time RT-PCR was performed with the ABI 7900 real-time PCR system (Applied Biosystems) using the following cycling parameters: 60°C for 10 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min, and a dissociation stage of 95°C for 15 s, 60°C for 1 min, 95°C for 15 s, and 60°C for 15 s. Primers were used to amplify a specific 100- to 120-bp fragment corresponding to a specific gene. The primers used for the IL-12p40 and β-actin genes are as follows: IL-12 forward, 5′ TGGTGTCTCCACTCATGG 3′; IL-12 reverse, 5′ AGCAGCAGATGTGAGTGG 3′; β-actin forward, 5′ AGGTGTGCACCT TTTATTGGTCTCAA 3′; β-actin reverse, 5′ TGTATGAAGGTTTGGTCT CCCT 3′. All data are presented as relative expression units after normalization to the β-actin gene. Measurements were conducted in triplicate. The differences in the relative expression of IL-12 were analyzed by analysis of variance (ANOVA) followed by Tukey's test ($P < 0.05$).

Fluorescence-activated cell sorter (FACS) analysis. For intracellular detection of IFN-γ and IL-17, splenocytes from C57BL/6, TLR2 KO, and TLR9 KO mice at 30 days postinfection with *M. avium* strain 2447 or 2-151 were adjusted to 1×10^6/well. The cells were stimulated with *M. avium* (MOI, 1:5) or ConA (5 μg/ml) at 37°C for 42 h, and brefeldin A (1 μg/well; Sigma) was added for the last 4 h. Cells were then stained for surface markers and intracellular cytokines. Briefly, cultured cells were incubated for 20 min with Fc-block anti-mouse CD16/32 (clone 93; eBioscience, San Diego, CA) in FACS buffer (0.15 M phosphate-buffered saline [PBS], 0.25% BSA, 1 mM NaN_3) and stained for surface markers using fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD4 (clone GK1.5; eBioscience, San Diego, CA). After 20 min, cells were washed, fixed using a 4% formaldehyde solution, and permeabilized with a 0.5% saponin solution in PBS. Cells were stained with phycoerythrin (PE)-conjugated anti-mouse IFN-γ (clone XMG1.2; BD Pharmingen, San Diego, CA) or PE-conjugated anti-mouse IL-17 (clone eBio18F10; eBioscience, San Diego, CA). After 30 min, cells were washed with permeabilization solution and resuspended in PBS. Cells (70,000 events) were collected using a FACScan flow cytometer (Becton Dickinson, San Jose, CA), and data were analyzed using the FlowJo Software (Tree Star, Ashland, OR). Further, BMDCs from wild-type and TLR9 KO, TLR2 KO, and MyD88 KO mice were infected for 24 h with *M. avium* 2447 (MOI, 5:1) and stained with FITC-conjugated anti-mouse CD11c (clone N418; eBioscience, San Diego, CA) plus PE-conjugated anti-mouse MHC class II (clone AF6-120.1; BD Pharmingen, San Diego, CA). Cells were then fixed, and acquisition (50,000 events) and data analysis were performed as described above. Nonstimulated BMDCs were used as negative controls.

Histopathological analysis. The medial lobes of the livers of *M. avium*-infected mice were collected at 30 and 100 days postinfection, fixed in 10% buffered formaldehyde solution, dehydrated, diaphanized, and embedded in paraffin. Four-micrometer-thick tissue sections were stained with hematoxylin and eosin (H&E). The total number of granulomas present in histological liver sections was determined using an Axiolab microscope (Carl Zeiss, Oberkochen, Germany) with a 10× objective lens. Digital images of the sides were captured at a resolution of 300 dots/in. using a scanner (HP Scanjet 2400; Hewlett-Packard, Loveland, CO), and the total area of each section was measured with the KS300 software connected to a Carl Zeiss (Oberkochen, Germany) image analyzer. The number of granulomas in each section was normalized for a 50-mm^2 tissue area. Furthermore, the lymphocytes and macrophages/epithelioid cells in 15 hepatic granulomas per animal were counted using the same microscope.

Statistical analysis. Statistically significant differences between the results obtained with KO mice and wild-type animals were evaluated by ANOVA followed by Tukey's *post hoc* test ($P < 0.05$).

RESULTS

Engagement of TLR9 in the control of *M. avium* infection. In order to determine the contribution of TLR9 to the host defense against *M. avium* infection *in vivo*, we have infected mice by the intravenous route and monitored the growth of *M. avium* in spleens, livers, and lungs. Hepatic and pulmonary bacterial burdens were significantly elevated in MyD88 KO,

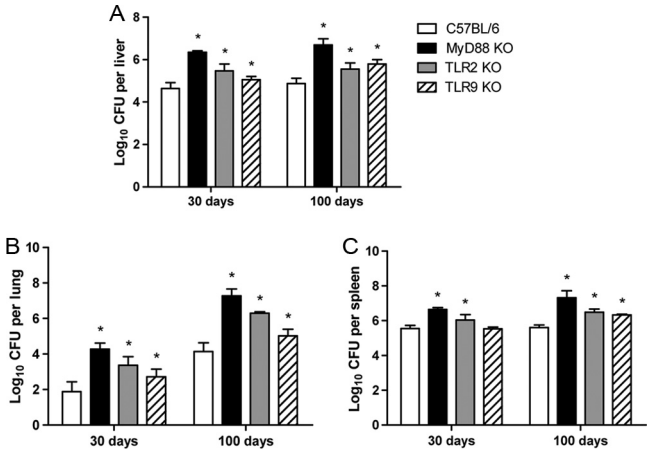

FIG. 1. Growth of *M. avium* in the livers (A), lungs (B), and spleens (C) of C57BL/6, MyD88 KO, TLR2 KO, and TLR9 KO mice. C57BL/6 and MyD88-, TLR2- and TLR9-deficient mice were intravenously inoculated with 10^6 CFU of *M. avium* 2447 ($n = 7$ mice per group). Bacterial loads in mouse organs were measured at 30 and 100 days postinfection. The significance of observed differences in CFU counts between C57BL/6 and KO mice were compared by ANOVA followed by Tukey's test (*, $P < 0.05$). The results shown are representative of two independent experiments.

TLR2 KO, and TLR9 KO mice at 30 and 100 days postinfection compared to those in C57BL/6 mice. Similar alterations in *M. avium* CFU counts were also observed in the spleens of TLR2 KO and MyD88 KO mice, but not in those of TLR9 KO mice, which exhibited increased CFU counts in spleens only at 100 days postinfection (Fig. 1).

Lack of TLR2 and TLR9 does not affect the production of proinflammatory cytokines by splenocytes stimulated *in vitro* with *M. avium*. To investigate whether TLR9 and TLR2 are involved in the production of proinflammatory cytokines following *M. avium* infection, total spleen cells obtained from infected KO mice were stimulated with live bacteria and levels of IFN-γ and TNF-α were measured by ELISA. The production of these cytokines was lower in MyD88 KO mice, but no differences were observed between TLR2 KO or TLR9 KO mouse cells and those of C57BL/6 mice at 30 days (Fig. 2) or 100 days postinfection (data not shown). Regarding the controls, TLR2 and MyD88 KO splenocytes did not respond to Pam3CSK4 and TLR9 and MyD88 KO cells were not activated by CpG ODN1826, as expected. To confirm the ELISA data, we performed flow cytometry analysis to determine the profile of $CD4^+$ T cells producing IFN-γ in TLR2 KO, TLR9 KO, and wild-type mice infected with SmT strain 2447 or 2-151. As demonstrated in Table 1, no difference between the numbers of Th1 cells from TLR9 or TLR2 KO mice and wild-type mice was detected, confirming the data obtained with splenocytes by ELISA. Additionally, the numbers of $CD4^+$ T cells expressing IL-17 were also determined, but again no statistically significant differences between TLR2 KO or TLR9 KO mice and C57BL/6 mice were detected (data not shown).

TLR9 deficiency does not influence *M. avium* growth in macrophages. To test the role of TLR9 in controlling *M. avium* growth in macrophages, we infected wild-type, TLR9 KO, or

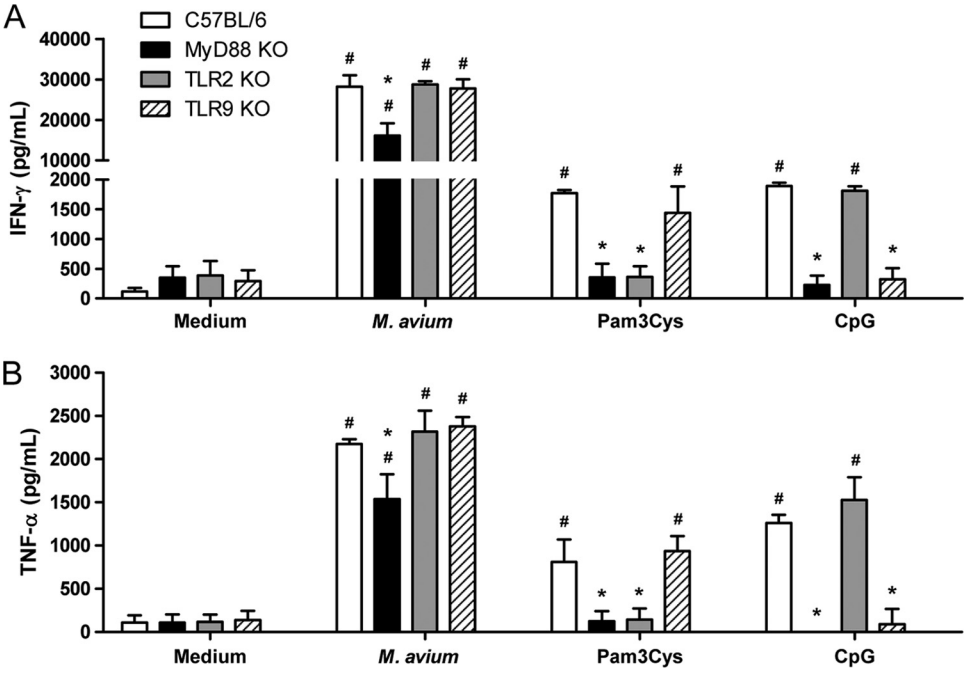

FIG. 2. Recall IFN-γ and TNF-α responses by spleen cells of infected animals are MyD88 dependent. Spleen cells (1×10^6) of C57BL/6, MyD88 KO, TLR2 KO, and TLR9 KO mice were cultured in the presence of *M. avium* 2447 (MOI, 5:1), Pam3CSK4 (1 μg/ml), CpG ODN1826 (1 μg/ml), or *E. coli* LPS (1 μg/ml, data not shown) at 30 days postinfection with *M. avium*. Levels of TNF-α and IFN-γ were measured by ELISA. Statistically significant differences between KO and C57BL/6 mice are indicated by an asterisk ($P < 0.05$), and those between unstimulated and stimulated spleen cells are indicated by the symbol # ($P < 0.05$).

TABLE 1. Percentages of $CD4^+$ T cells producing IFN-γ on day 30 after infection with *M. avium* strain 2447 or 2-151

M. avium and mouse strains	Mean % of $CD4^+$ T cells producing IFN-γ ± SD		
	Medium	*M. avium*	ConA
2447			
C57BL/6	3.27 ± 1.04	10.67 ± 1.85[a]	19.90 ± 5.70[a]
TLR2 KO	2.79 ± 0.38	10.60 ± 1.71[a]	13.15 ± 3.06[a]
TLR9 KO	3.91 ± 0.24	11.20 ± 0.57[a]	18.55 ± 1.87[a]
2-151			
C57BL/6	3.82 ± 0.51	8.94 ± 0.98[a]	12.93 ± 2.05[a]
TLR2 KO	2.90 ± 0.54	7.54 ± 1.49[a]	9.98 ± 2.85[a]
TLR9 KO	4.10 ± 0.73	7.62 ± 0.81[a]	12.33 ± 2.73[a]

[a] Statistically significant difference between unstimulated and stimulated cells ($P < 0.05$).

MyD88 KO BMMs with *M. avium* 2447 SmT and measured the intracellular growth of the bacterium for 7 days. The growth of *M. avium* inside MyD88 KO macrophages (0.27 log) was slightly greater than that observed in wild-type macrophages, with no difference between TLR9 KO and C57BL/6 mouse macrophages (Fig. 3).

***M. avium* DNA induces the production of proinflammatory cytokines through a TLR9-dependent pathway.** To assess whether *M. avium* DNA is a potent stimulus for TLR9, BMMs from C57BL/6 and TLR9 KO mice were stimulated with *M. avium* DNA and levels of proinflammatory cytokines were measured. *M. avium* DNA induced TNF-α and IL-12p40 production by BMMs from wild-type animals, but these cytokines were not detected in the cultures of BMMs from TLR9 KO mice (Fig. 4). These results indicate that the production of proinflammatory cytokines induced by *M. avium* DNA is dependent on TLR9 signaling.

IL-12 and TNF-α production by antigen-presenting cells in response to *M. avium* is primarily regulated by TLR2. BMMs and BMDCs were generated and stimulated *in vitro* with *M. avium* to evaluate whether the enhanced susceptibility of TLR9 KO or TLR2 KO mice to infection was associated with impaired production of cytokines by antigen-presenting cells. Macrophages derived from both MyD88 KO and TLR2 KO

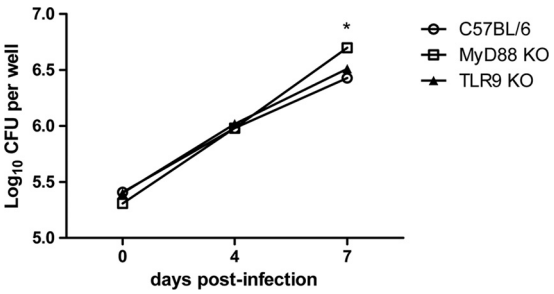

FIG. 3. MyD88, but not TLR9, controls *M. avium* growth in macrophages. BMMs from C57BL/6, MyD88 KO, and TLR9 KO mice were infected with *M. avium* 2447 (MOI, 1:2) and incubated for 7 days to measure the intracellular growth of bacteria. For each time point, three independent culture wells containing macrophages were used. Results are the means ± standard deviations of three wells. Statistical analysis was performed using ANOVA followed by Tukey's test (*, $P < 0.05$).

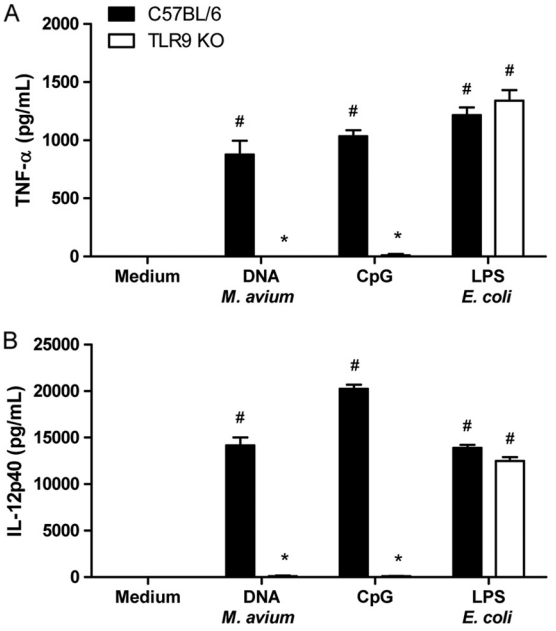

FIG. 4. IL-12 and TNF-α production induced by DNA of *M. avium* in BMMs is TLR9 dependent. BMMs from C57BL/6 and TLR9 KO mice were stimulated with *M. avium* 2447 DNA (10 μg/ml), CpG ODN1826 (1 μg/ml), or *E. coli* LPS (1 μg/ml) for 24 h. Levels of TNF-α (A) and IL-12 (B) in culture supernatants were determined by ELISA. Statistically significant differences between KO and C57BL/6 mice are indicated by an asterisk ($P < 0.05$), and those between unstimulated and stimulated spleen cells are indicated by the symbol # ($P < 0.05$). Results are means ± standard deviations of experiments performed with three animals. Data shown are representative of two different experiments.

mice displayed significantly reduced IL-12p40 and TNF-α production in response to live *M. avium*. In contrast, high levels of these cytokines were produced by TLR9-deficient macrophages and by cells derived from wild-type animals (Fig. 5). Additionally, BMDCs from TLR9 KO mice also induced the same amounts of IL-12p40 and TNF-α as wild-type BMDCs and the production of these cytokines was markedly reduced in the absence of TLR2 and MyD88 (Fig. 6). Regarding the controls, TLR2 and MyD88 KO macrophages or DCs did not respond to Pam3CSK4 and TLR9 and MyD88 KO cells were not activated by CpG ODN1826, as expected. In order to test later IL-12 gene expression, IL-12 transcripts were measured by real-time PCR in macrophages at 3, 5, and 7 days postinfection with *M. avium*. This experiment revealed that in a time course evaluation, TLR2, but not TLR9, is required for IL-12 mRNA expression at 3, 5, and 7 days following infection (Fig. 7).

TLR2, but not TLR9, regulates MHC class II expression by DCs infected with *M. avium*. In order to characterize the role of MyD88, TLR2, and TLR9 in DC maturation, we infected BMDCs with *M. avium* 2447 and analyzed the cell surface expression of MHC class II high molecules on $CD11c^+$ cells by flow cytometry. TLR9 KO DCs showed enhanced expression of MHC class II similar to that of wild-type DCs. However, lower expression of these molecules were observed in MyD88 KO and TLR2 KO DCs than in wild-type mice, indicating that *M. avium*-induced MHC class II expression is dependent on

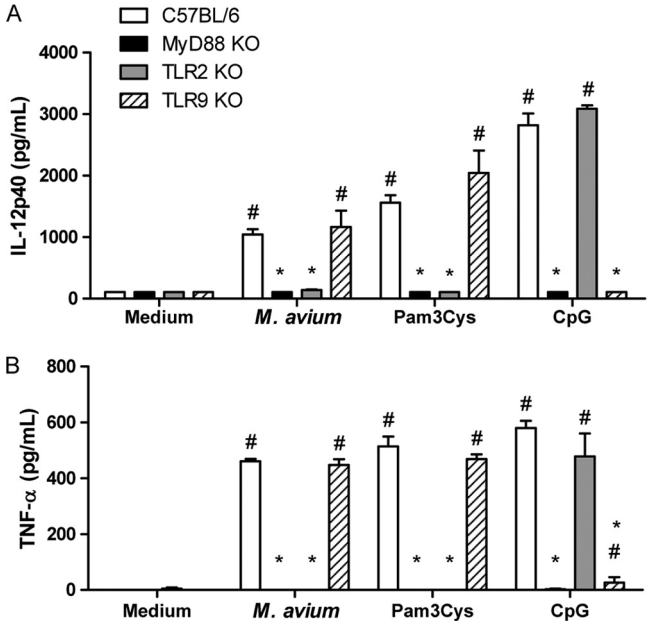

FIG. 5. *M. avium* induction of IL-12 and TNF-α production by BMMs is TLR9 independent. BMMs from C57BL/6, MyD88 KO, TLR2 KO, and TLR9 KO mice were stimulated with *M. avium* 2447 (MOI, 5:1), 1 μg/ml Pam3CSK4, 1 μg/ml CpG ODN1826, or 1 μg/ml *E. coli* LPS (data not shown) for 24 h. Levels of IL-12 (A) and TNF-α (B) in culture supernatants were assayed by ELISA. Statistically significant differences between KO and C57BL/6 mice are indicated by an asterisk ($P < 0.05$), and those between unstimulated and stimulated spleen cells are indicated by the symbol # ($P < 0.05$). Results are means ± standard deviations of experiments performed with three animals. Data shown are representative of two different experiments.

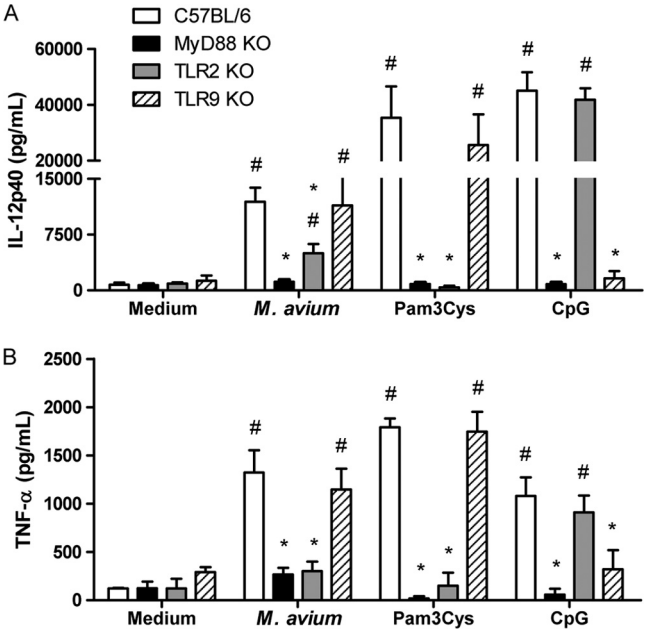

FIG. 6. Lack of effect of TLR9 deficiency on IL-12 and TNF-α production by BMDCs stimulated with live *M. avium*. Levels of IL-12 (A) and TNF-α (B) were measured by ELISA in supernatants of BMDCs derived from C57BL/6, MyD88 KO, TLR2 KO, and TLR9 KO mice following antigen stimulation. Statistically significant differences between KO and C57BL/6 mice are indicated by an asterisk ($P < 0.05$), and those between unstimulated and stimulated spleen cells are indicated by the symbol # ($P < 0.05$). Results are means ± standard deviations of experiments performed with three animals. Data shown are representative of two different experiments.

TLR2 signaling (Fig. 8). The same experiment was performed using macrophages with similar results (data not shown).

Lack of TLR2 or TLR9 reduced the number of macrophages, epithelioid cells, and lymphocytes in *M. avium*-induced granuloma. To assess whether the increased bacterial burden in KO mice alters liver pathology, the number and cell composition of granulomas in this organ were determined. As demonstrated in Fig. 9, we observed differences in leukocyte composition in the liver at 30 days postinfection between TLR9 KO, TLR2 KO, and MyD88 KO mice and wild-type mice. The numbers of macrophages and epithelioid cells were greater than those of lymphocytes in all of the animals; however, the numbers of all of the cell types studied were lower in KO mice than in C57BL/6 mice (Fig. 9E). The most prominent reduction in leukocyte numbers, as well as the granuloma size, as observed in histological sections, was detected in MyD88 KO mice (Fig. 9A to D). Additionally, MyD88 KO mice showed more hepatic granulomas than C57BL/6 mice did; however, TLR9 KO and TLR2 KO mice did not demonstrate altered granuloma counts (Fig. 10).

DISCUSSION

The participation of TLRs in the recognition of *M. avium* and induction of protective immunity against infection by this pathogen has been suggested by previous studies on TLR2 KO and MyD88 KO mice (6, 10). However, no studies so far have addressed the role of TLR9 in *M. avium* infection. The highly enhanced susceptibility of animals deficient in the adaptor molecule MyD88 was indicative that multiple TLRs might be acting in concert to efficiently trigger full host resistance to *M.*

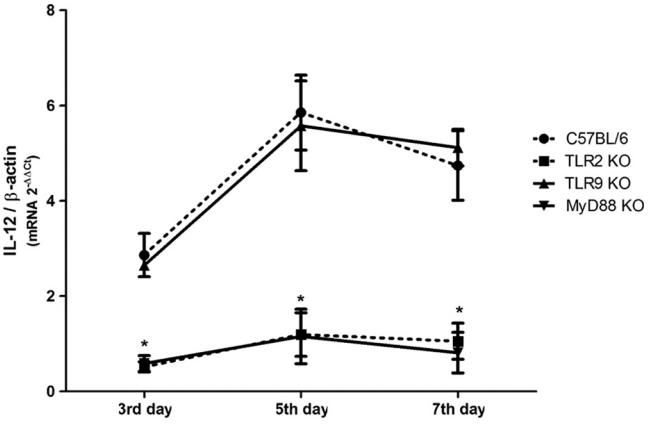

FIG. 7. *M. avium* infection induces IL-12 expression in C57BL/6 and TLR9 KO mouse macrophages but not in MyD88 KO or TLR2 KO mouse cells. At 3, 5, and 7 days after strain 2447 infection, total RNA was extracted and the relative expression of IL-12 was assessed by real-time RT-PCR. The data are presented as means ± standard deviations of relative expression units after normalization to the β-actin gene. Statistically significant differences between KO and C57BL/6 mice are indicated by an asterisk ($P < 0.05$). This experiment was performed in triplicate.

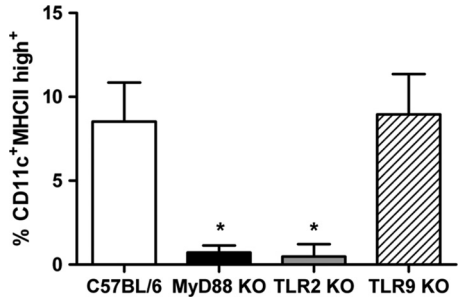

FIG. 8. *M. avium*-induced MHC class II expression by BMDCs requires TLR2 but not TLR9. Flow cytometry analysis of BMDCs derived from C57BL/6, MyD88 KO, TLR2 KO, and TLR9 KO mice infected with *M. avium* 2447 (MOI, 5:1). $CD11c^+$ cells were analyzed for the expression of surface MHC class II high molecules. Results are expressed as percentages of those of double-positive cells. The values observed for nonstimulated BMDCs (negative control) were subtracted from the results. Statistically significant differences ($P < 0.05$) between KO and wild-type BMDCs are indicated by an asterisk. The experiments were carried out in triplicate, and two independent experiments were performed with similar results.

avium (6). Herein, we observed that *M. avium* DNA induces IL-12 and TNF-α in a TLR9-dependent manner. Additionally, we determined that TLR9 contributes to the control of infection *in vivo* by this bacterium, since bacterial burdens were augmented in organs of infected TLR9 KO mice. Strikingly, production of Th1 proinflammatory cytokines was apparently not altered in either the splenic cells of these animals or TLR9-deficient macrophages (BMMs) and BMDCs infected *in vitro* with *M. avium*, indicating that the involvement of this receptor in the control of infection may not be related to the induction of Th1 responses.

Recent studies have reported the role played by TLR9 during infection by mycobacterial pathogens other than *M. avium* (2, 30). It has been proposed that this receptor is required for efficient recognition of *M. tuberculosis* and *Mycobacterium bovis* and that it participates in mechanisms such as full activation of DCs, induction of proinflammatory cytokines, regulation of pulmonary granulomatous responses, and ultimately control of

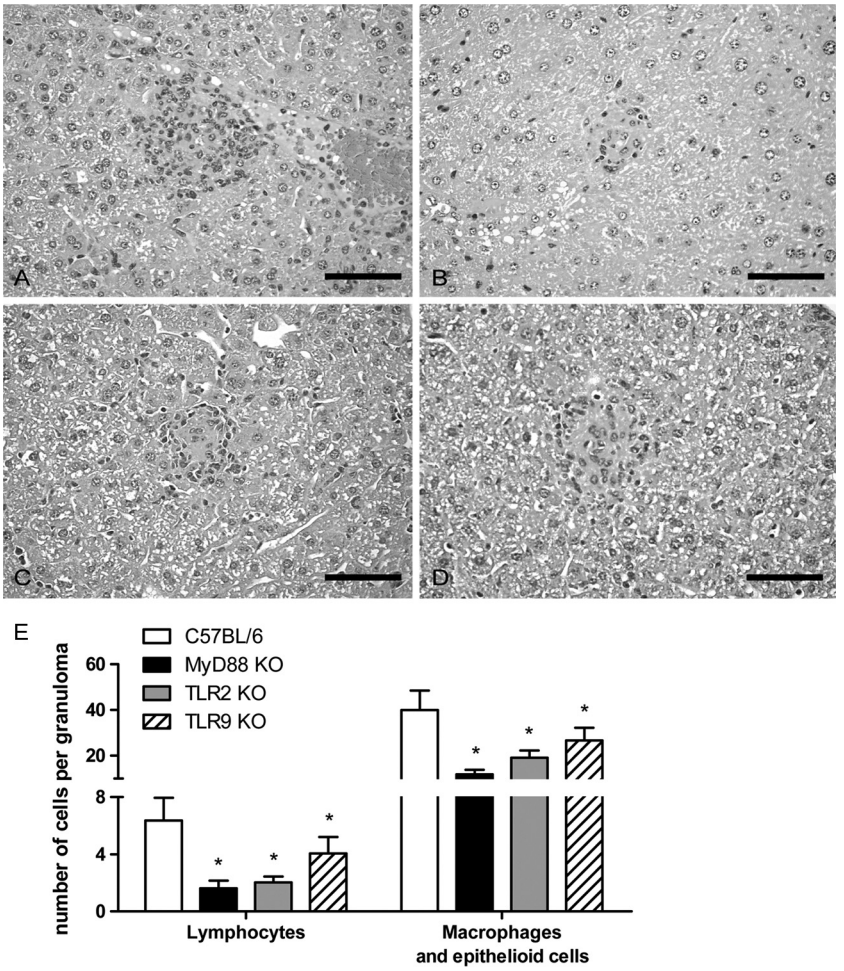

FIG. 9. Histopathology and cell composition of hepatic granulomas of C57BL/6, MyD88 KO, TLR2 KO, and TLR9 KO mice infected with *M. avium*. Formalin-fixed, paraffin-embedded hepatic tissue sections from mice infected for 30 days were stained with H&E. Representative granulomas from infected C57BL/6 (A), MyD88 KO (B), TLR2 KO (C), and TLR9 KO (D) mice are shown. Bars, 30 μm. (E) To perform cell counts, liver fragments were collected from infected C57BL/6, MyD88 KO, TLR2 KO, and TLR9 KO mice at 30 days postinfection. Tissue sections were processed and stained with H&E. The lymphocytes and macrophages/epithelioid cells in 15 hepatic granulomas per animal were counted using a light microscope. Statistically significant differences ($P < 0.05$) between KO and C57BL/6 mice are indicated by an asterisk. Results are means ± standard deviations of five animals per group.

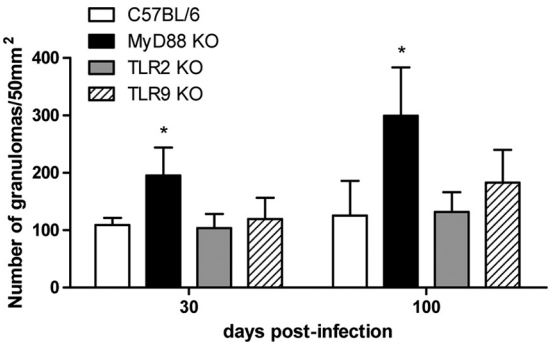

FIG. 10. Increased numbers of hepatic granulomas during *M. avium* infection in MyD88 KO mice. Liver fragments were collected from infected C57BL/6, MyD88 KO, TLR2 KO, and TLR9 KO mice at 30 and 100 days postinfection. Tissue sections were processed and stained with H&E. The hepatic granulomas were counted using a light microscope. Images of each section were captured, and the area of the section was measured using software. The number of granulomas was normalized for 50 mm^2 of hepatic tissue. Statistically significant differences ($P < 0.05$) between KO and C57BL/6 mice are indicated by an asterisk. Results are means ± standard deviations of five animals per group.

mycobacterial infections (2, 16, 17, 30). More recently, Chen et al. (4) have reported the role of TLR9 in host resistance to infection, demonstrating that inhibition of TLR9 prevents *M. tuberculosis*-induced apoptosis. A scenario in which multiple TLRs collaborate in triggering innate immunity reactions and protective Th1 responses is strongly suggested by these previous studies, TLR2 being a major synergistic receptor for the recognition of mycobacteria (2, 30). A contrasting view has been expressed by Hölscher et al. (14), who proposed that involvement of TLRs, including TLR2, TLR9, and MyD88 signaling, in *M. tuberculosis* pattern recognition has little impact on the induction of Th1 responses. Furthermore, they claimed that MyD88, but not TLR2 or TLR9, is necessary for the induction of protective immune responses triggered by macrophage effector mechanisms. Herein, our observation on the role of TLR9 during *in vivo M. avium* infection is partially in agreement with other studies of *M. tuberculosis* and *M. bovis* (2, 30), since TLR9 KO mice were not able to control the infection by this pathogen with the same efficiency as wild-type mice (Fig. 1). In contrast, we observed that TLR9 is not required for the induction of IFN-γ, IL-17, TNF-α, and IL-12p40 following infection with pathogenic bacteria, as reported by Hölscher et al. (14) for *M. tuberculosis*. Therefore, the definitive mechanism by which this receptor contributes to the clearance of this bacterium remains to be elucidated.

On the other hand, our findings regarding the involvement of TLR2 in host resistance to *M. avium* are consistent with a role for this receptor in proinflammatory cytokine induction in both infected BMMs and BMDCs measured by ELISA or by real-time RT-PCR in macrophages (Fig. 5 to 7). Previous reports have already demonstrated the requirement of IL-12, IFN-γ, and TNF-α for resistance to *M. avium* infection (1). We have observed that TLR2 deficiency does not alter IFN-γ production by *in vitro* stimulated splenic cells at 30 days postinfection, and this result is in accordance with those of Gomes and colleagues (10). However, the production of TNF-α and IL-12p40 by *M. avium*-infected TLR2 KO BMMs and BMDCs was dramatically reduced to levels similar to those of MyD88 KO cells under the conditions evaluated in the present study. This finding led us to speculate that *M. avium* induced IFN-γ production *in vivo* by splenocytes is independent of TLR2-mediated IL-12 synthesis. A similar observation for *M. avium*-infected TLR2 KO BMMs was also reported by Feng et al. (6) and Gomes et al. (11) for macrophages but not DCs.

A granuloma is a characteristic pathophysiological feature induced by mycobacterial infections, and it is a hallmark of the protective immune response (29). However, the mechanisms involved on granuloma formation are not clear. Previously, Ito et al. (17) have demonstrated that TLR9 plays an important role in maintaining the Th1 granulomatous response during *M. tuberculosis* infection. More recently, the same group found that the larger lung granulomas detected in TLR9 KO mice are due to impaired delta-like 4 Notch ligand expression and therefore a reduced Th17 cytokine profile (16). In our study looking at liver granulomas during *M. avium* infection, we observed that TLR9, TLR2, and MyD88 molecules are involved in granuloma cell composition. Reduced numbers of macrophages, epithelioid cells, and lymphocytes were detected in TLR9 KO, TLR2 KO, and MyD88 KO mouse granulomas than in those of C57BL/6 mice. In contrast, TLR9 or TLR2 had no influence in the increased numbers of liver granulomas after *M. avium* infection observed in MyD88 KO mice. The higher numbers of granulomas in MyD88 KO mice might be related to the greater number of *M. avium* CFU observed in the mouse liver. Regarding cellular content, the reduced phenotype observed in MyD88 KO mice is possibly due to diminished IFN-γ and TNF-α production required for granuloma formation.

Once inside macrophages, pathogens use a large array of strategies to evade or counteract host immune responses. For example, they can diminish or abrogate their antigen presentation capacity, thus reducing T-cell-mediated immune responses. Additionally, $CD4^+$ T cells and MHC class II molecules required for $CD4^+$ T-cell responses are critical to host resistance to *M. tuberculosis* (25). In this study, we have observed that *M. avium*-induced MHC class II expression on the surface of BMDCs is dependent on TLR2, but not TLR9, signaling. These data suggest that DC maturation and probably antigen presentation are regulated partially by TLR2. Furthermore, we speculate that antigen presentation can be possibly manipulated by *M. avium* to its advantage since it has been shown that prolonged TLR2 signaling induced by *M. tuberculosis* lipoproteins (TLR2 agonists) results in the inhibition of MHC class II molecule expression and antigen presentation by *M. tuberculosis*-infected macrophages (13).

Mycobacterial lipids are important pathogen-associated molecular patterns recognized by host pattern recognition receptors. Trehalose 6,6′-dimycolate (TDM; cord factor) is the most abundant lipid of the *M. tuberculosis* cell wall, and this molecule signals through MyD88 in macrophages, producing IL-1β, IL-6, and TNF-α (9). More recently, Bowdish et al. (3) have identified a class A scavenger receptor, MARCO (macrophage receptor with collagenous structure), as a receptor for *M. tuberculosis* TDM. They indicated that MARCO, which is expressed by certain types of macrophages, is utilized to tether TDM molecules to the macrophage surface and to activate the TLR2 signaling pathway, resulting in the activation of NF-κB.

MARCO-expressing macrophages secrete proinflammatory cytokines, including TNF-α, more effectively in response to TDM due to cooperation between MARCO and TLR2/CD14 than MARCO-deficient macrophages do. These findings imply that the scavenger receptor plays an essential role as a receptor for TDM and identify MARCO as a novel component required for TLR signaling. Additionally, Werninghaus et al. (31) have shown that TDM activated macrophages and DCs via the Syk-Car9-Bcl10-Malt1 pathway to induce specific innate activation events distinct from TLR-mediated signaling pathways and induced the production of Th1 and Th17 in response to an *M. tuberculosis* subunit vaccine and partial protection against *M. tuberculosis* infection. Notably, TDM has been detected in nontuberculous mycobacteria, including *M. avium* complex species, which rarely form true cords, suggesting that this glycolipid is not sufficient for this property (27). Indeed, *M. tuberculosis* and *M. avium* cord factors markedly differ in mycolic acid subclasses and molecular species composition (8). These glycolipid structural differences could explain why the pattern of TLR-mediated activation of cellular functions of macrophages and DCs in response *to M. avium* is different from that elicited by *M. tuberculosis* stimulation. Species-specific compositions of TLR2-stimulating lipid components of mycobacterial organisms, including lipoglycans such as TDM, lipoarabinomannan, glycopeptidolipids and lipomannan; phosphatidyl-*myo*-inositol mannosides; and lipoproteins, may determine the mode of activation of cellular functions of macrophages and DCs in response to mycobacterial stimulation.

The central role of MyD88 in immunity to *M. avium* and *M. tuberculosis* contrasts with the more limited involvement of TLRs, suggesting that other MyD88-dependent pathways and molecules are pivotal for mycobacterial control. In the case of *M. tuberculosis* infection models, IL-1 and IL-18 may be those molecules, as shown by the increased susceptibility of IL-1R KO, IL-1β KO, and IL-18 KO mice to tuberculosis (7, 18, 22, 26). IL-1R KO mice were already studied with regard to susceptibility to *M. avium* by Feng and colleagues (6) and shown not to differ from the respective controls. However, these animals carry the closely associated allele of *Nramp1* (*Slc11A1*) which confers innate resistance to *M. avium*, making them unsuitable to address the role of this cytokine receptor in the context of natural susceptibility to infection. It is curious that, despite their increased susceptibility to tuberculosis, mice with disruptions in IL-1 and IL-18 signaling show uncompromised Th1 responses during infection with *M. tuberculosis* (7, 22, 26). Our observation that lack of MyD88 significantly alters the granulomatous response, an observation also reported by a study with *M. tuberculosis* infection in IL-1R-deficient mice (18), may suggest that IL-1 family cytokines may protect via optimal granuloma assembly.

In summary, we have demonstrated that TLR9 participates in full host resistance to *M. avium* infection by a mechanism independent of Th1 cell development. The fact that TLR2 also contributes to the control of infection and the production of proinflammatory cytokines in macrophages and DCs suggests a possible collaborative role for these TLRs in protective immunity to *M. avium*.

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