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Nucleotide-Binding Oligomerization Domain Protein 2-Deficient Mice Control Infection with *Mycobacterium tuberculosis*▽

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Nucleotide-binding oligomerization domain proteins (NODs) are modular cytoplasmic proteins implicated in the recognition of peptidoglycan-derived molecules. NOD2 has recently been shown to be important for host cell cytokine responses to *Mycobacterium tuberculosis*, to synergize with Toll-like receptor 2 (TLR2) in mediating these responses, and thus to serve as a nonredundant recognition receptor for *M. tuberculosis*. Here, we demonstrate that macrophages and dendritic cells from NOD2-deficient mice were impaired in the production of proinflammatory cytokines and nitric oxide following infection with live, virulent *M. tuberculosis*. Mycolylarabinogalactan peptidoglycan (PGN), the cell wall core of *M. tuberculosis*, stimulated macrophages to release tumor necrosis factor (TNF) and interleukin-12p40 in a partially NOD2-dependent manner, and *M. tuberculosis* PGN required NOD2 for the optimal induction of TNF. However, NOD2-deficient mice were no more susceptible to infection with virulent *M. tuberculosis* than wild-type mice: they controlled the replication of *M. tuberculosis* in lung, spleen, and liver as well as wild-type mice, and both genotypes displayed similar lung pathologies. In addition, mice doubly deficient for NOD2 and TLR2 were similarly able to control an *M. tuberculosis* infection. Thus, NOD2 appears to participate in the recognition of *M. tuberculosis* by antigen-presenting cells in vitro yet is dispensable for the control of the pathogen during in vivo infection.

Mycobacterium tuberculosis has successfully infected one-third of the world's population and threatens to kill 10% of infected individuals during the course of their lifetime (1, 11). Few genetic loci for mycobacterial resistance or susceptibility have been identified, and it is a daunting task to uncover correlates of protection against tuberculosis (TB). Innate and adaptive immune responses are required for host defense that leads to the control of mycobacterial replication within macrophages. The infected macrophages are part of an organized granuloma consisting of multiple immune cells including macrophages, dendritic cells (DCs), and T and B lymphocytes. The interaction of *M. tuberculosis* with phagocytes results in the production of proinflammatory cytokines and chemokines and is crucial for coordinated innate and adaptive immune responses and thus for effective granuloma formation (10).

M. tuberculosis interacts with phagocytes via a variety of receptors (8). Although Toll-like receptors (TLRs) on macrophages and DCs are important for the recognition of *M. tuberculosis* (2, 31), *M. tuberculosis* activates these cells via both TLR-dependent and TLR-independent pathways. For example, global gene expression analysis revealed that *M. tuberculosis* induces gene expression in murine bone marrow-derived macrophages (BMMs) chiefly independently of MyD88, the central intracellular adaptor of TLRs (33). Expression of some proinflammatory cytokines such as interleukin-1 (IL-1) and IL-6 depends predominantly on TLR2-mediated recognition in macrophages and DCs (19, 33). However, many proinflammatory mediators including interferon-inducible protein 10, inducible nitric oxide (NO) synthase (iNOS), immune-responsive gene 1, and RANTES are induced by *M. tuberculosis* in BMMs in the absence of TLR2 and TLR4. IL-12p40 expression in *M. tuberculosis*-infected DCs is induced in a TLR2- and TLR4-independent manner; instead, its production requires TLR9 in vitro and in vivo (2, 19). Cooperation between different pattern recognition receptors also contributes to the host response to *M. tuberculosis*, which is demonstrated by the observation that TLR2 and TLR9 act synergistically to mediate resistance to *M. tuberculosis* infection (2).

TLRs are not the only receptors involved in sensing microbial infection. Nucleotide-binding oligomerization domain proteins (NODs) are members of an emerging family that have been implicated in the intracellular recognition of bacterial components (16). NOD2 recognizes muramyl dipeptide (MDP), a component of peptidoglycan (PGN) from both gram-positive and gram-negative bacteria (12–14, 17). NOD2 contains a carboxyl-terminal leucine-rich repeat domain, a central nucleotide-binding oligomerization domain, and two amino-terminal caspase recruitment domains (CARDs) (18). Following exposure to MDP, NOD2 is hypothesized to interact with the serine/threonine kinase Rip2/RICK/CARDIAK via CARD-CARD binding (21, 29, 36). RICK directly activates the NF-κB pathway through the activation of the IκB kinase complex, leading to the degradation of IκBα and the release of NF-κB (29). Data focusing on the immunological relevance of NOD2 are just starting to emerge. Mutations in human *CARD15*, encoding NOD2, are partially associated with susceptibility to several familial inflammatory diseases including Crohn's disease, Blau syndrome, and early-onset sarcoidosis

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(15, 20, 25, 28). NOD2 may therefore be important for regulating inflammatory responses (16). Further evidence that NOD2 might be involved in the control of inflammation comes from studies with NOD2-deficient (*Card15*$^{-/-}$) mice that produced higher serum IL-12 levels than wild-type mice when systemically challenged with the TLR2 agonist PGN (40). NOD2 was also found to be required for the control of gastric infection with *Listeria monocytogenes* in mice yet dispensable for the control of systemic infection (22). Thus, NOD2 specifically protected against bacterial infection in the intestine, where it was required for the expression of a subgroup of intestinal antimicrobial peptides.

M. tuberculosis is an intraphagosomal pathogen; however, mycobacterial proteins and cell wall lipids access the cytosol, where they encounter intracellular molecules to modulate the host cell response (3, 4, 27). Indeed, a recent study showed that tumor necrosis factor alpha (TNF-α) production induced by sonicated *M. tuberculosis* in murine peritoneal macrophages was partially NOD2 dependent (9). However, the role of NOD2 in mediating immune responses that are required for the control of infection with live, virulent *M. tuberculosis* has not been tested. We used NOD2-deficient (*Card15*$^{-/-}$) BMMs and DCs to show that NOD2 is required for the optimal production of proinflammatory cytokines and NO in response to *M. tuberculosis* infection in vitro. However, infection of *Card15*$^{-/-}$ mice and *Card15*$^{-/-}$ *Tlr2*$^{-/-}$ mice with virulent *M. tuberculosis* revealed that the impaired cellular responses did not result in increased susceptibility to TB. Thus, NOD2 participates in the innate recognition of *M. tuberculosis*, yet in vivo, redundant systems that mediate an effective immune response to *M. tuberculosis* even in the absence of NOD2 seem to exist.

MATERIALS AND METHODS

Mice. *Card15*$^{-/-}$ mice were generated as previously described and used on a C57BL/6 background ($n = 5$ backcross generations) (30). *Card15*$^{-/-}$ *Tlr2*$^{-/-}$ mice, also on a C57BL/6 background ($n = 5$ backcross generations), were generated as described previously (41). Mice were housed under specific-pathogen-free conditions.

Macrophage preparation. Bone marrow cells from 8- to 10-week-old mice were flushed from femurs and differentiated into macrophages for 7 days in Dulbecco's modified Eagle medium supplemented with 20% L-cell medium, 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, and 10 mM HEPES. Cells were fed with 25% fresh medium on day 4. After 7 days in culture, BMMs were washed with phosphate-buffered saline (PBS) and seeded into tissue culture plates in Dulbecco's modified Eagle's medium containing 10% L-cell medium, 10% fetal bovine serum, 2 mM L-glutamine, and 1 mM sodium pyruvate. This results in a nearly pure macrophage population as assessed by morphology and cell surface staining of CD14, F4/80, FcγRII/III, and major histocompatibility complex class II, the latter after gamma interferon (IFN-γ) activation. Where indicated, 10 ng/ml mouse IFN-γ (R&D Systems) was added. Sixteen hours later, the cells were infected with a single-cell suspension of *M. tuberculosis* obtained from early-log-phase cultures (7).

DC preparation. Bone marrow-derived DCs were prepared as previously described (23). Briefly, bone marrow was flushed out from the femur and tibia, and 2×10^6 bone marrow cells were seeded into 10-cm petri dishes in 10 ml of RPMI containing 10% fetal calf serum (HyClone Laboratories, Logan, UT) and supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), glutamine (2 mM), 2-mercaptoethanol (50 μM), and 20 ng/ml murine recombinant granulocyte-monocyte colony-stimulating factor (Peprotech Inc., Rocky Hill, NJ). On day 3, an additional 10 ml of complete medium containing granulocyte-monocyte colony-stimulating factor was added to the cultures. On days 6 and 8, the cultures were fed by changing 50% of the medium. Nonadherent cells were harvested on day 10. Resultant nonadherent cells were typically >70% CD11c$^+$ CD11b$^+$ as determined by fluorescence-activated cell sorter analysis.

Bacteria and microbial stimuli. *M. tuberculosis* strains H37Rv, Erdman, and 1254 (ATCC 51910) were used as indicated. Bacterium-derived stimuli included 10-ng/ml lipopolysaccharide (LPS) from *Salmonella enterica* serovar Friedenau H909 (a kind gift by H. Brade, Research Center Borstel, Germany), 10-μg/ml MDP (Sigma), 2- to 15-μg/ml *M. tuberculosis* PGN, and 10-μg/ml mycoarabinogalactan PGN (mAGP).

qRT-PCR. Four or 24 h postinfection, macrophages were lysed in TRIzol, and total RNA was isolated as described previously (33). Three hundred nanograms of RNA was transcribed into cDNA with gene-specific primers in 20 ml using 50 U of Moloney murine leukemia virus reverse transcriptase (Perkin-Elmer). To control for the presence of DNA in RNA preparations, a parallel reaction without reverse transcriptase was performed. cDNA was diluted to 100 μl and 5 μl and used for quantitative real-time PCR (qRT-PCR). PCR was performed in a volume of 15 μl on the ABI PRISM 7900HT sequence detection system (Perkin-Elmer) as previously described (33). Control reactions were subjected to the same qRT-PCR reaction. The sequences of primers and probes were previously described (33).

Quantification of cytokine and chemokine release by ELISA. BMMs from *Card15*$^{+/+}$ and *Card15*$^{-/-}$ mice were seeded into 48-well plates at 2×10^5 cells/well. Cells were infected with live *M. tuberculosis* cells at a multiplicity of infection (MOI) of 3. Supernatants were collected 24 h postinfection for TNF-α and 48 to 72 h postinfection for IL-12/IL-23p40 quantification. Concentrations of mouse TNF-α, IL-12/IL-23p40, RANTES (CCL-5), and monocyte chemoattractant protein 1 (MCP-1) (CCL-2) were determined by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (R&D Systems and BD OptEIA, respectively). For bone marrow DC-derived supernatants, sandwich ELISA was performed using the following antibody pairs from BD Pharmingen (San Diego, CA): C15.6 and C17.8 (biotinylated) for IL-12p40, JES5-2A5 and JES5-16E3 (biotinylated) for IL-10, G281-2626 and MP6-XT3 (biotinylated) for TNF-α, and 9A5 and C17.8 (biotinylated) for IL-12p70. An OptEIA mouse IL-6 kit (BD Pharmingen, San Diego, CA) was used for IL-6 measurements.

Mouse infections. Mice that were 8 to 10 weeks of age were infected with single-cell suspensions of logarithmic-phase cultures of *M. tuberculosis* by aerosol using an inhalation exposure system (Glas-Col). Animals were exposed for 40 min to an aerosol produced by nebulizing 5 ml of a bacterial suspension in PBS at a concentration of 2×10^7 bacilli/ml for low-dose challenge and 1×10^8 bacilli/ml for an intermediate-dose challenge. To obtain a high-dose challenge (2,000 CFU/lung), 5 ml of bacterial suspension (2×10^7 bacilli/ml) prepared in PBS containing 0.05% Tween 80 was nebulized.

Mice were euthanized by inhalation of CO_2, and their lungs, spleen, and liver were aseptically removed and homogenized in 4 or 5 ml of PBS containing 0.05% Tween 80. To confirm the infectious dose, on day 1 postinfection, 1.6 ml of 4-ml lung homogenates was plated onto 10% oleic acid–albumin–dextrose–catalase and 0.5% glycerol-enriched 7H11 (Difco) plates. Plates were incubated at 37°C, and CFU were enumerated 14 to 21 days later. Mice were sacrificed at the indicated times postinfection; their lungs, spleens, and livers were aseptically removed and homogenized in PBS containing 0.05% Tween 80; and serial dilutions were plated onto enriched 7H11 plates for CFU enumeration. The upper left lobe of infected lungs was fixed in 10% formalin to analyze pathology in sections stained with hematoxylin and eosin. All animal studies were approved by the Institutional Animal Care and Use Committee of Weill Medical College of Cornell University.

RESULTS

Impaired NO production by NOD2-deficient macrophages in response to *M. tuberculosis* infection. The generation of reactive nitrogen intermediates is a crucial innate immune defense mechanism against infection with *M. tuberculosis*. Therefore, we tested the ability of *Card15*$^{-/-}$ macrophages to generate reactive nitrogen intermediates following infection with virulent *M. tuberculosis*. Nitrite accumulation in supernatants of *M. tuberculosis*-infected BMMs was assayed 24 h postinfection. IFN-γ-primed BMMs from *Card15*$^{-/-}$ mice produced over 50% less NO than *Card15*$^{+/+}$ macrophages following *M. tuberculosis* infection (Fig. 1A). Infection of cells that were not primed with IFN-γ did not result in detectable nitrite accumulation as previously described (7). *Card15*$^{-/-}$ macro-

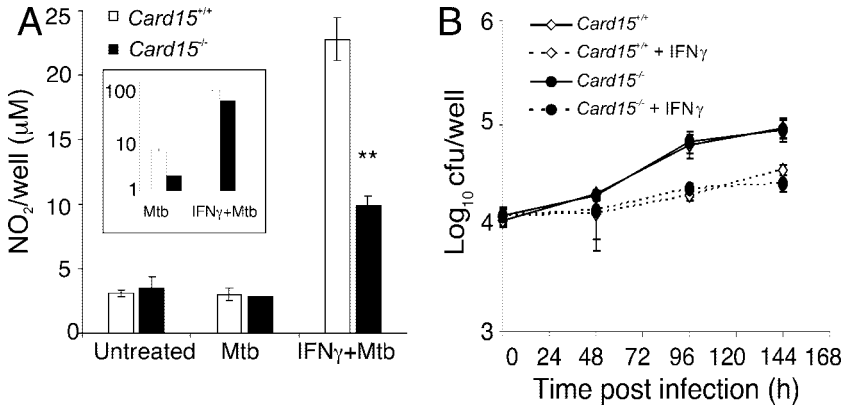

FIG. 1. Antimycobacterial activity of *Card15*$^{-/-}$ macrophages. A total of 2×10^5 BMMs from *Card15*$^{+/+}$ (white bars and symbols) and *Card15*$^{-/-}$ (black bars and symbols) mice were seeded per well of a 48-well tissue culture plate in the presence or absence of 10 ng/ml IFN-γ. Sixteen hours after stimulation with IFN-γ, macrophages were infected with *M. tuberculosis* at an MOI of 3, and nitrite accumulation in cell supernatants was measured 24 h postinfection by Griess assay (A). The inset in A shows the quantification of iNOS transcript levels normalized to 1×10^4 GAPDH (glyceraldehyde-3-phosphate dehydrogenase) transcripts at the same time point. Intracellular replication of *M. tuberculosis* was assessed by plating lysates of infected cells onto 7H11 agar plates at the indicated time points (B). Data are representative of three independent experiments, each done with triplicate wells per group. **, $P < 0.01$.

phages also induced two- to threefold-lower levels of iNOS transcript than *Card15*$^{+/+}$ macrophages in response to *M. tuberculosis* alone or *M. tuberculosis* in the presence of IFN-γ (Fig. 1A, inset). These data suggest that NOD2 is required for optimal iNOS mRNA expression and NO production in response to infection with *M. tuberculosis*.

NOD2 is not required for control of *M. tuberculosis* replication in macrophages in vitro. To test if impaired NO production by *Card15*$^{-/-}$ macrophages resulted in an impaired control of intracellular mycobacterial replication, we infected macrophages with *M. tuberculosis* and measured intracellular survival by CFU (Fig. 1B). The uptake of *M. tuberculosis* was not affected by the lack of NOD2. Surprisingly, there was also no defect in the control of mycobacterial growth or survival in resting and IFN-γ-primed cells by *Card15*$^{-/-}$ macrophages compared to wild-type macrophages up to 6 days postinfection. Thus, despite reduced NO production, NOD2-deficient cells were still able to control the replication of intracellular *M. tuberculosis*.

Optimal TNF-α, IL-12p40, and RANTES production by macrophages in response to live *M. tuberculosis* requires NOD2. The impaired NO release of *Card15*$^{-/-}$ macrophages suggested that NOD2 is involved in macrophage activation by *M. tuberculosis*. We therefore tested whether the production of proinflammatory cytokines by *M. tuberculosis*-infected macrophages required NOD2. TNF-α levels in supernatants of *M. tuberculosis*-infected *Card15*$^{-/-}$ macrophages were twofold reduced compared to *Card15*$^{+/+}$ macrophages in both the presence and absence of IFN-γ (Fig. 2A). IL-12p40 levels in culture supernatants of *Card15*$^{-/-}$ macrophages were three- and fivefold lower than those of their wild-type counterparts upon *M. tuberculosis* infection in the presence and absence of IFN-γ, respectively (Fig. 2B). Activation with IFN-γ enhanced cytokine production by *Card15*$^{-/-}$ macrophages although not to levels produced by IFN-γ-activated *Card15*$^{+/+}$ macrophages. To test if the absence of NOD2 altered chemokine production in response to *M. tuberculosis* infection, we measured concentrations of RANTES and MCP-1 in supernatants of *M. tuberculosis*-infected macrophages. We found that the concentration of RANTES was twofold reduced in supernatants of *Card15*$^{-/-}$ macrophages compared to their wild-type counterparts (Fig. 2C); however, MCP-1 levels were not reproducibly altered in NOD2-deficient macrophages (data not shown). These data indicate that NOD2 is involved in macrophage

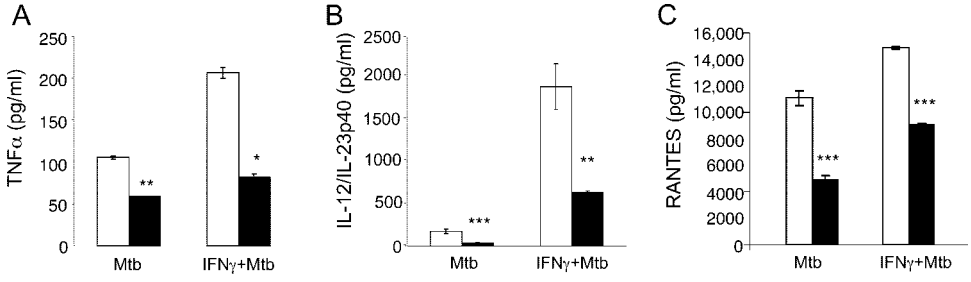

FIG. 2. Reduced cytokine production by *Card15*$^{-/-}$ macrophages in response to *M. tuberculosis*. A total of 2×10^5 BMMs were seeded per well of a 48-well tissue culture plate in the presence or absence of 10 ng/ml IFN-γ. Sixteen hours later, macrophages were infected with *M. tuberculosis* (Mtb) at an MOI of 3. TNF-α levels at 24 h (A), IL-12p40 levels at 48 h (B), and RANTES levels at 24 h (C) postinfection were determined by ELISA. Data are representative of three independent experiments, each done with triplicate wells per group. *, $P < 0.05$; **, $P < 0.01$.

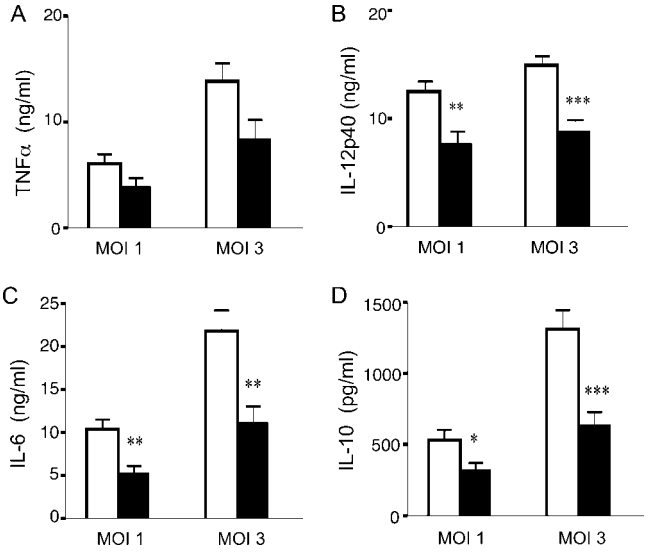

FIG. 3. Reduced cytokine production by *Card15*$^{-/-}$ DCs in response to *M. tuberculosis*. A total of 2.5×10^5 DCs derived from the bone marrow of *Card15*$^{+/+}$ and *Card15*$^{-/-}$ mice were infected with *M. tuberculosis* at MOIs of 1 and 3. Levels of TNF-α (A), IL-12-p40 (B), IL-6 (C), and IL-10 (D) in cell supernatants were determined by ELISA at 20 h postinfection. Data are representative of three independent experiments, each done with triplicate wells per group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

activation by *M. tuberculosis*, but not all macrophage responses require NOD2.

NOD2 is required for cytokine production by DCs in response to live *M. tuberculosis*. We next examined whether NOD2 also contributes to optimal cytokine production in DCs following infection with *M. tuberculosis*. DCs derived from wild-type and *Card15*$^{-/-}$ mice were infected with *M. tuberculosis* at MOIs of 1 and 3; 16 h later, the supernatants were tested for the presence of IL-12p40, IL-12p70, IL-6, IL-10, and TNF-α. As shown in Fig. 3, at both MOIs tested, there was a significant reduction in IL-12p40, IL-12p70 (data not shown), IL-6, and IL-10 in the absence of NOD2. The lack of NOD2 had a less drastic effect on TNF-α secretion by DCs. Together, the data suggest that in the absence of NOD2, DC release reduced amounts of all cytokines tested, suggesting that the NOD2 recognition system participates in the response to *M. tuberculosis*. However, in contrast to macrophages, the production of RANTES by *M. tuberculosis*-infected DCs did not require NOD2 (data not shown).

mAGP-induced cytokine production is partially NOD2 dependent. *M. tuberculosis* cell wall components are potent inducers of cytokines in macrophages, and this activity can be mimicked by stimulating cells with dead bacteria. We first tested if NOD2-dependent macrophage activation required live *M. tuberculosis* and measured NO, TNF, and IL-12p40 levels in BMMs stimulated with heat-killed *M. tuberculosis*. The production of the three proinflammatory mediators was reduced in *Card15*$^{-/-}$ macrophages compared to *Card15*$^{+/+}$ macrophages (Fig. 4A), suggesting that a heat-stable component of *M. tuberculosis* activated BMMs in a NOD2-dependent manner. To further identify the microbial component(s) that stimulates NOD2 to mediate optimal NO and cytokine production, we treated macrophages with different cell wall components from *M. tuberculosis*, including purified mAGP and PGN, synthetic MDP, and LPS from *S. enterica* as a positive control for macrophage responsiveness because NOD2 is not required for the LPS pathway (22, 30).

When *Card15*$^{+/+}$ and *Card15*$^{-/-}$ macrophages were stimulated with 10 μg/ml mAGP and 5 μg/ml PGN from *M. tuberculosis*, PGN induced only marginal NO release in both *Card15*$^{+/+}$ and *Card15*$^{-/-}$ cells primed with IFN-γ (Fig. 4B). By contrast, mAGP activated IFN-γ-primed macrophages to release significant amounts of NO, but we observed no significant differences in NO production between wild-type and *Card15*$^{-/-}$ macrophages (Fig. 4B). However, stimulation with the NOD2 agonist MDP resulted in diminished NO production in *Card15*$^{-/-}$ macrophages when cells were primed with IFN-γ, consistent with previously published data (38) concerning the NOD2 dependence on MDP-induced NO production.

MDP stimulated TNF-α and IL-12p40 production in wild-type macrophages in both the absence and presence of IFN-γ in a NOD2-dependent manner (Fig. 4C and D, left), consistent with previous data from three different NOD2 loss-of-function mutant strains that are all similarly deficient in MDP responsiveness (22, 24, 30). mAGP-treated *Card15*$^{-/-}$ macrophages also produced lower TNF-α ($P < 0.005$) and IL-12p40 ($P < 0.001$) levels than wild-type macrophages. In the presence of IFN-γ, there was a similar trend of reduced TNF-α and IL-12p40 production by *Card15*$^{-/-}$ macrophages; however, this difference was not statistically significant (Fig. 4C and D, middle).

PGN is a component of mAGP that contains the NOD2 agonist MDP. TNF-α production in response to 5 μg/ml and 10 μg/ml *M. tuberculosis* PGN was two- to threefold reduced in the absence of NOD2 in resting and IFN-γ-primed macrophages (Fig. 4C, right). This difference subsided when the PGN concentration was increased to 15 μg/ml. PGN also induced IL-12p40 production, but this was not dependent on NOD2 at any of the concentrations tested (Fig. 4D, right).

Taken together, these results suggest that the mycobacterial cell wall fragments stimulate macrophages partially via NOD2 to induce cytokine production, but the degree and type of response depend on the structure and composition of the cell wall fragments used, highlighting the complexity of stimulation and response pathways for *M. tuberculosis*.

NOD2 is not required for in vivo control of *M. tuberculosis*. To test if impaired cytokine production by *Card15*$^{-/-}$ macrophages and DCs affects the ability of mice to control mycobacterial replication in vivo, we infected mice by aerosol with a low dose (~35 CFU/lung), an intermediate dose (~220 CFU/lung), and a high dose (~1,500 CFU/lung) of *M. tuberculosis* and monitored growth of *M. tuberculosis* over time. We used two different *M. tuberculosis* strains, H37Rv and the clinical isolate strain 1254. None of these infections resulted in a significant difference in bacterial burdens in lungs, spleens, or livers of *Card15*$^{+/+}$ and *Card15*$^{-/-}$ mice at weeks 1, 3, and 8 postinfection (Fig. 5A and B and data not shown). Histological analysis revealed no obvious differences in lung pathology between the two genotypes at 8 weeks postinfection (Fig. 5E and F). We measured iNOS mRNA levels by qRT-PCR in infected mouse lungs at week 1 and week 3 after low-dose infection and found no difference in iNOS mRNA expression (data not

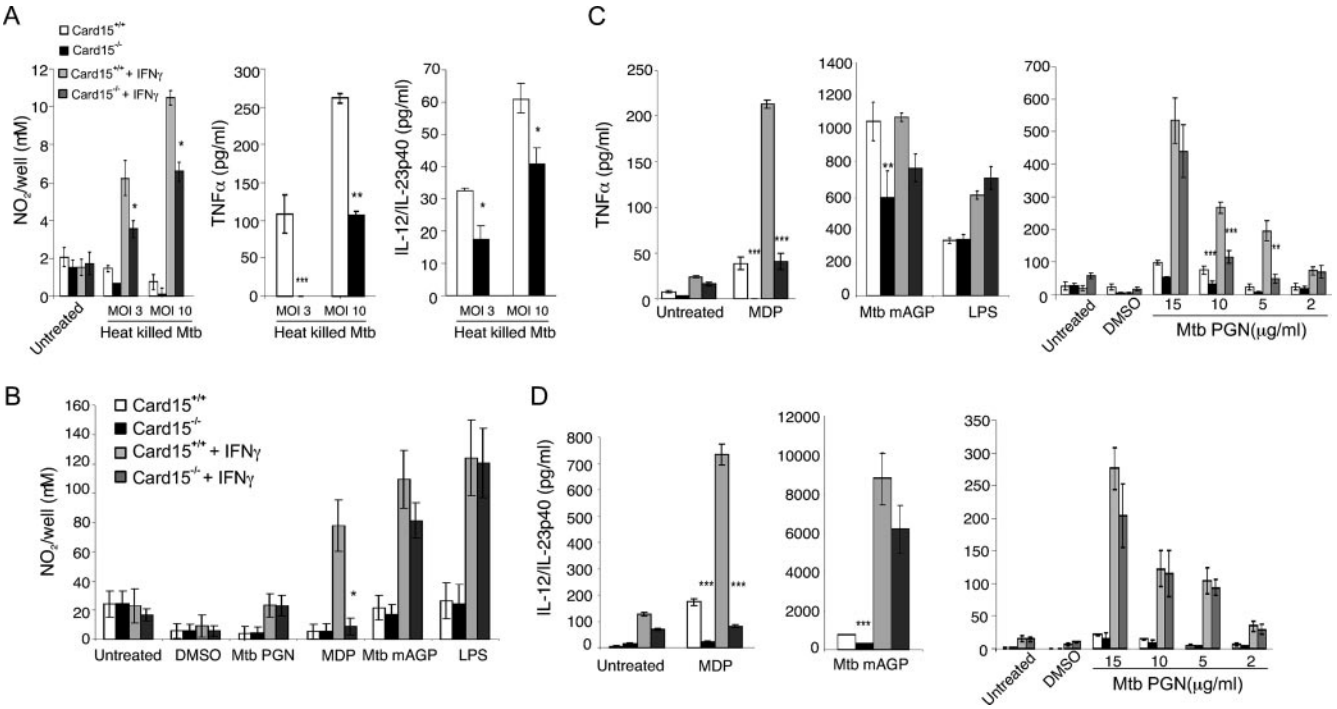

FIG. 4. Role of NOD2 in MDP-, mAGP-, and PGN-induced macrophage responses. A total of 2×10^5 BMMs were seeded per well of 48-well tissue culture plates in the presence or absence of 10 ng/ml IFN-γ. Sixteen hours later, macrophages were stimulated, and macrophage responses were measured. (A) Nitrite accumulation at 72 h, TNF-α levels at 24 h, and IL-12-p40 levels at 48 h postinfection with heat-killed *M. tuberculosis* (Mtb). (B) Nitrite accumulation after 72 h of stimulation with 5 μg/ml *M. tuberculosis* PGN, 10 μg/ml *M. tuberculosis* mAGP cell wall core, 10 μg/ml MDP, or 10 ng/ml LPS. (C and D) TNF-α levels at 24 h (C) and IL-12-p40 levels at 72 h (D) after stimulation with 10 μg/ml MDP, 10 μg/ml *M. tuberculosis* mAGP, 10 ng/ml LPS, and *M. tuberculosis* PGN at the indicated concentrations. Data are representative of two to three independent experiments, each done with triplicate cultures per group. Data from PGN-stimulated BMMs are from two independent experiments, each done in triplicate. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

shown). Serum IL-12/IL-23p40 levels were also not different except at week 1 postinfection, when they were twofold higher in *Card15*$^{-/-}$ mice ($P = 0.0089$ for low-dose infection, $P = 0.019$ for intermediate-dose infection, and $P = 0.085$ for high-dose infection) (Fig. 5C and D and data not shown). Taken together, these results demonstrate that despite the impaired ability of *CARD15*$^{-/-}$ cells to produce TNF-α, IL-12/IL-23p40, IL-6, IL-10, RANTES, and NO in response to *M. tuberculosis* in vitro, *Card15*$^{-/-}$ mice were able to control an infection with virulent *M. tuberculosis* as well as wild-type mice.

To test for a potential redundancy between NOD2- and TLR2-mediated signaling, we infected *Card15*$^{-/-}$/*Tlr2*$^{-/-}$ mice with *M. tuberculosis*. As shown in Fig. 6, bacterial numbers were indistinguishable at 3 weeks postinfection in lungs from *Card15*$^{+/+}$/*Tlr2*$^{+/+}$ and *Card15*$^{-/-}$/*Tlr2*$^{-/-}$ mice. At 8 and 17 weeks postinfection, CFU were twofold and threefold higher in lungs from *Card15*$^{-/-}$/*Tlr2*$^{-/-}$ mice than in lungs from *Card15*$^{+/+}$/*Tlr2*$^{+/+}$ mice. However, bacterial numbers in lungs from mice of both genotypes declined from week 3 postinfection to the 17-week time point, indicating that *Card15*$^{-/-}$/*Tlr2*$^{-/-}$ mice were able to control the infection similarly to wild-type mice.

DISCUSSION

NOD2 participates in a signaling pathway that mediates recognition and responses to PGN-derived MDP (12). Because PGN from both gram-negative and gram-positive bacteria as well as that from acid-fast mycobacteria contain MDP, NOD2 is thought to function as a sensor of most bacteria, although the mechanisms involved in the direct or indirect MDP-NOD2 interaction are unresolved. Our goal was to investigate the role of NOD2 in the recognition of live, virulent *M. tuberculosis* by antigen-presenting cells and to test whether NOD2 is required for host resistance against *M. tuberculosis* infection. We demonstrated that macrophages and DCs lacking NOD2 are impaired in their ability to induce proinflammatory cytokines and NO upon infection with *M. tuberculosis*. NO is critical for the host defense against *M. tuberculosis*, and NO production by macrophages in response to mycobacterial lipoproteins or intact bacteria can occur through TLR-dependent and independent routes (5, 32, 33, 37). These data suggested that NOD2 is one of the recognition molecules required for an optimal NO response. Although NO production in NOD2-deficient cells was reduced, there was a clear distinction between different subcellular products with regard to NOD2 dependence to induce NO. NO release in response to MDP was NOD2 dependent, consistent with previous data (38), while *M. tuberculosis* PGN and mAGP did not require NOD2 to induce NO in BMMs. Mycobacterial lipoproteins mediate NO production via TLR2 (5, 37); however, the induction of iNOS and NO release in response to intact, live *M. tuberculosis* is largely TLR2 independent (32). Collec-

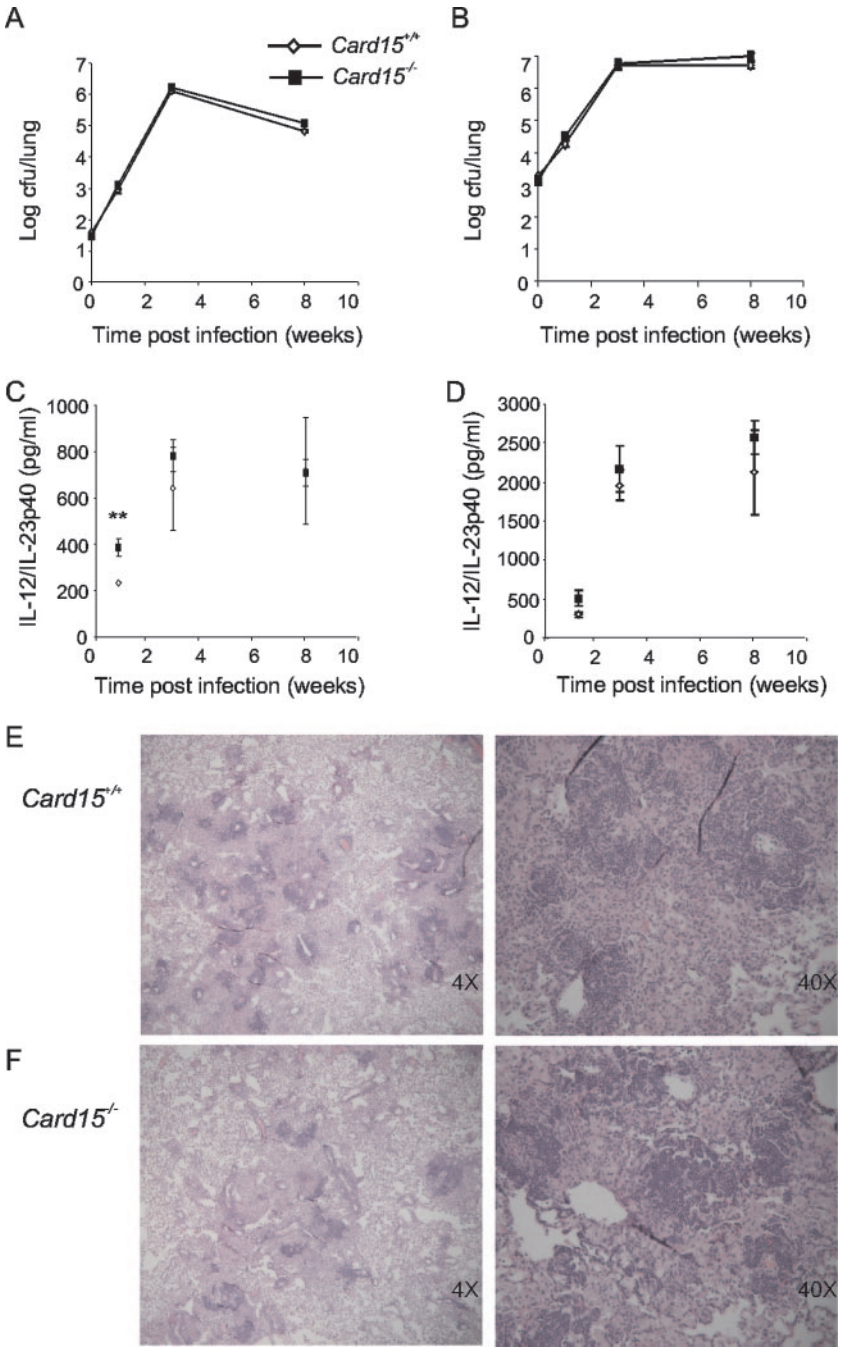

FIG. 5. *Card15*$^{-/-}$ mice control infection with *M. tuberculosis*. *Card15*$^{+/+}$ and *Card15*$^{-/-}$ mice were infected by aerosol with a low dose (35 CFU) of a clinical isolate, *M. tuberculosis* 1254 (A), or with a high dose (1,500 CFU) of strain H37Rv (B). At the indicated time points, four to five mice of each group were sacrificed, and bacteria were enumerated in the lungs (A and B), spleens (not shown), and livers (not shown). Serum IL-12p40 levels after low-dose (C) and high-dose (D) infection were measured by ELISA. Error bars indicate standard deviations. **, $P < 0.01$. The upper left lobes of lungs from *Card15*$^{+/+}$ (E) and *Card15*$^{-/-}$ (F) mice infected with a low dose of *M. tuberculosis* were formalin fixed and paraffin embedded at 8 weeks postinfection. Tissue sections were stained with hematoxylin and eosin. Representative sections from one mouse per genotype out of four are shown.

tively, these data suggest that macrophages have evolved independent pathways for iNOS induction and that *M. tuberculosis* possesses multiple agonists that are capable of stimulating these pathways.

Human mononuclear cells from patients with Crohn's disease expressing a mutated NOD2 and peritoneal macrophages from mice lacking NOD2 responded with impaired TNF-α and IL-10 production when stimulated with sonicated *M. tuberculosis* (9). We found that TNF-α, IL-12p40, and RANTES production by BMMs and IL-12p40, IL-6, and IL-10 production by DCs upon infection with live, virulent *M. tuberculosis* were reduced in the absence of NOD2. Furthermore, MDP and

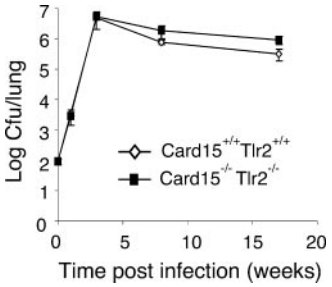

FIG. 6. Control of mycobacterial growth in *Card15*$^{-/-}$ *Tlr2*$^{-/-}$ mice. *Card15*$^{+/+}$ *Tlr2*$^{+/+}$ and *Card15*$^{-/-}$ *Tlr2*$^{-/-}$ mice were infected with a low dose of *M. tuberculosis* H37Rv (100 CFU). Bacterial counts in lungs were determined at weeks 1, 3, 8, and 17 postinfection ($n = 4$ mice per group). Error bars indicate standard deviations.

mAGP, the mycobacterial cell wall core, mimicked the dependence on NOD2 for both TNF-α and IL-12p40 production. Treatment with mycobacterial PGN, a component of mAGP, also resulted in decreased levels of TNF-α in NOD2-deficient cells. IL-12p40 production was not NOD2 dependent when *M. tuberculosis* PGN instead of mAGP was used to stimulate macrophages. This may be the result of additional immunomodulatory features of mAGP such as mycolic acids or arabinogalactan. PGN is only one part of the highly cross-linked macromolecular structure of mAGP, and the response to such a complex agonist may not reflect the specificity of any one component. Watanabe et al. demonstrated enhanced Th1 responses, including IL-12p40 production, by PGN-treated splenocytes and splenocyte-derived antigen-presenting cells from *Card15*$^{-/-}$ mice compared to wild-type spleen macrophages (40, 41). We did not observe increased IL-12p40 production by bone marrow-derived NOD2-deficient macrophages after stimulation with PGN from *M. tuberculosis* in vitro. Perhaps the macrophage source is the reason for the different results. This is supported by the observation that IL-12p40 levels in the serum of *M. tuberculosis*-infected *Card15*$^{-/-}$ mice were increased 7 days postinfection relative to *Card15*$^{+/+}$ mice, similar to the increased serum IL-12p40 levels in *Card15*$^{-/-}$ mice injected with PGN (40). Serum IL-12p40 is likely produced by circulating and splenic monocytes that may resemble the response of PGN-activated spleen macrophages from *Card15*$^{-/-}$ mice described previously by Watanabe et al. (40, 41).

The impaired in vitro responses of NOD2-deficient antigen-presenting cells to infection with *M. tuberculosis* suggested that NOD2 may be required for host defense against *M. tuberculosis*. However, NOD2-deficient mice were no more susceptible to *M. tuberculosis* infection with low and high doses than wild-type mice up to 8 weeks postinfection. The early difference in serum IL-12p40 levels between *M. tuberculosis*-infected *Card15*$^{+/+}$ and *Card15*$^{-/-}$ mice subsided at later time points of infection and did not result in any detectable difference with respect to bacterial load, cellular recruitment, and lung pathology. Thus, despite impaired immune responses of NOD2-deficient cells in vitro, NOD2 was dispensable for the control of *M. tuberculosis* in vivo during the acute and early persistent phases of the infection. Mycobacteria possess both TLR and NOD2 agonists, and combinatory dual signaling through TLRs and NOD2 may result in synergistic host cell activation (9, 22, 39). However, mice that were doubly deficient for NOD2 and TLR2 controlled the replication of virulent *M. tuberculosis* as well as wild-type mice in the acute phase of the infection, and during the chronic phase, bacterial titers even declined slowly. Infection with high but not low doses of *M. tuberculosis* resulted in a loss of resistance in *Tlr2*$^{-/-}$ mice compared to wild-type mice (6, 31, 32, 35). We cannot rule out that *Card15*$^{-/-}$/*Tlr2*$^{-/-}$ mice are more susceptible to infection with a high dose of *M. tuberculosis* than wild-type mice. It is also possible that a potential role of NOD2 in the pathogenesis of TB may become apparent in a situation of compromised immunity, for example, during the reactivation of a chronic infection. Future experiments are required to address these questions. Our current data suggest that redundant signaling systems can compensate for the loss of TLR2 and NOD2 following a low-dose aerosol challenge in mice.

Taken together, the findings of this study provide strong evidence that NOD2 is not essential for host defense in murine TB models. Recent genotyping studies of TB patients showed no significant association of six single-nucleotide polymorphisms in the leucine-rich repeat domain of NOD2 or of eight single-nucleotide polymorphisms in the *Card15* promoter region with pulmonary TB in two different African populations, indicating that, at least in the populations studied, *Card15* is not a major susceptibility gene for TB (26, 34).

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